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Regulating Translation can Promote Different Stem Cell Fates in the Cyst Lineage of the *Drosophila* Testis Niche

Author: Freya Storer

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Masters of Research in the Faculty of Biomedical Sciences.

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Abstract

Stem cells have the ability to self-renew and differentiate. The present project investigated what happens in a stem cell as it differentiates to enable it to switch fate. The *Drosophila* testis model contains a niche, known as the hub that provides self-renewal signals and supports two stem cell populations, somatic cyst stem cells (CySCs) and germline stem cells (GSCs). Differentiation is considered a passive process, due to a lack of self-renewal signals, once stem cells exit the niche. Previous research in GSCs and haematopoeitic stem cells (HSCs) shows that translation rates increase in differentiating cells and that the increase is necessary for differentiation to occur. Other research also shows that differentiation in the cyst lineage is regulated by increasing PI3K/Tor pathway activity, of which translation is a known target. Translation is largely regulated at the initiation stage and can be initiated in both a cap-dependent and cap-independent manner. Cap-dependent translation requires the binding of eukaryotic initiation factor complex F (eIF4F) to the m^7G 5' cap of an mRNA strand, to recruit ribosomal subunits and initiate translation. Cap-independent methods, such as Internal ribosomal entry site (IRES)-associated translation rely on internal structures to recruit the ribosome independently. Initiation factors such as eIF3 and eIF2 are used in both. To test the role of translation initiation in CySC maintenance and differentiation, I conducted a screen to knock down translation initiation factors by RNAi. Strikingly, different initiation factors had different requirements during CySC differentiation: while knockdown of components of the cap-binding eIF4F complex led to a loss of self-renewal, knockdown of other translation initiation factors led to a lack of differentiation. These results indicate that cap-dependent initiation is required for CySC self-renewal, but dispensable for differentiation and suggest that initiation modes switch during differentiation.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .....  ..... DATE: 07/01/19

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Declaration of programmes used to generate Figures and Tables: *Introduction figures were taken from selected, referenced, published work. Results figures were constructed using ImageJ software and edited using Microsoft Powerpoint 2011. Graphs were constructed in Microsoft Excel 2011. Tables were constructed in Microsoft Word 2011.*

1.0 Introduction

1.1 Project Introduction

Stem cells are specialist cells that have a unique ability to give rise to themselves and to differentiating daughter cells, over the lifetime of an organism. They maintain adult tissue homeostasis by replenishing depleted cells, either through natural turnover (such as in skin or gut tissue) or in cases of injury. How they are able to both maintain themselves and give rise to differentiating cells is one of the most important basic questions in biology.

1.1.1 The importance of studying stem cells

Stem cell research is an exciting and important area of study due the opportunities to harness the potential of stem cells to regenerate and assist in the repair of damaged tissue. Stem cell therapy is already a successful form of treatment for various conditions, ranging from neurodegenerative diseases to cardiac problems (Henning, 2011; Sakthiswary and Raymond, 2012). Such treatment makes use of our understanding of how stem cells operate and how their fate is regulated. The cell-fate decision in stem cells is still poorly understood therefore continuing research in this area is important to improve therapies.

1.1.2 The potential for self-renewal and differentiation

Tissue homeostasis by balancing stem cell self-renewal and differentiation, having the capacity to adopt two different cell fates with different gene expression programmes (Enver *et al.*, 2009; Amoyel and Bach, 2012). It is still poorly understood how this process is regulated and specifically how a stem cell can entirely change its programme of expressed genes, from promoting self-renewal to promoting differentiation. The two main models that explain cell-fate decision include a transcription-regulated model (Moignard and Göttgens, 2014) and an epigenetic model (Okazaki and Maltepe, 2006), each regulating the expression of genes associated with different cell fates. The focus of the present project is on whether the regulation of protein synthesis/translation mediates gene expression and, by extension, whether it plays a role in the decision to adopt stem or differentiated cell fate.

1.1.3 Previous research in translation as a regulator of fate

Previous research has shown that protein synthesis rates play a role in stem cell differentiation. A 2014 study compared the translation rates of haematopoietic stem cells (HSCs) and restricted haematopoietic progenitors (RHPs). They found that less protein was synthesized by HSCs compared to their differentiating counterparts (Signer *et al.*, 2014). The study found that reducing ribosome function impaired HSC function, suggesting a regulatory role for translation in stem cell fate. They also saw that increasing PI3K/Tor activity through Pten loss, led to loss of the long-term potential of HSCs, and that reducing ribosome function in the Pten mutants rescued that phenotype (Signer *et al.*, 2014). Sanchez *et al.* investigated the effect of regulating protein synthesis in *Drosophila* germline stem cells (GSCs) to find a similar result in 2016. The study involved an RNA interference (RNAi) screen, which revealed a role for ribosome assembly factors in regulating stem cell cytokinesis. They suggested that protein synthesis modulated the transition from a self-renewing stem cell to a differentiating cell (Sanchez *et al.*, 2016). Another study in germline stem cells suggested that protein synthesis is essential in regulating GSC homeostasis (Yu *et al.*, 2016). The group performed an RNAi screen of 221 genes, which resulted in highlighting certain mRNA splicing and protein translation factors to be crucial for GSC maintenance and differentiation. Knocking down protein degradation genes in cyst cells led to over-proliferation of germ cells, resulting in testis tumours (Yu *et al.*, 2016). Knocking down mRNA splicing genes led to GSC self-renewal defects (Yu *et al.*, 2016). These few studies have suggested a role for translation in regulating stem cell fate and that increasing translation rates is necessary for differentiation to occur. This has founded the interest of the present project to investigate the role of translation further.

1.2 Protein Synthesis

Eukaryotic protein synthesis, also referred to as translation, is a vital biological process, which converts messenger RNA into polypeptides and eventually fully-folded, functional proteins. The mechanism consists of four main processes; initiation, elongation, termination and recycling. However, the most highly regulated process, involving multiple factors, is translation initiation.

1.2.1 Canonical/cap-dependent translation initiation

Regulating translation is crucial for healthy cell growth and proliferation (Sonenberg and Hinnebusch, 2009a). Translation is almost exclusively regulated at the initiation stage, where large complexes form at the N7-methylated Guanosine 5' cap (m^7G 5' cap) of messenger ribonucleic acids (mRNAs) (Lasko *et al.*, 2005). Most eukaryotic mRNAs contain an m^7G 5' cap (cap) structure, linked to the first mRNA nucleotide via triphosphate linkage to the 5' end. The m^7G 5' cap serves to recruit cellular proteins, including translation initiation factors, during cap-dependent initiation (Ramanathan, Robb and Chan, 2016). It also mediates other functions including pre-mRNA processing and nuclear export (Ramanathan, Robb and Chan, 2016). During cap-dependent translation initiation, eukaryotic initiation factor 4E (eIF4E) binds directly to the cap, ultimately positioning the ribosome close to the 5' terminus. Eukaryotic initiation factor 4A (eIF4A) is recruited and binds eIF4E, followed by eukaryotic initiation factor 4G (eIF4G) which acts as a scaffold protein between eIF4E and eIF4A. Altogether, the factors form a complex known as eIF4F. eIF4G also binds poly A binding protein (pAbp), which binds the poly A tail at the 3' terminus of the mRNA. This stabilises the mRNA molecule for translation. Eukaryotic initiation factor 5 (eIF5), eukaryotic initiation factor 1 and 1A (eIF1/1A) and eukaryotic initiation factor 3 (eIF3) bind the 40S ribosomal subunit. This causes the ribosomal subunit to bind with the so-called ternary complex, which consists of an activated transfer ribonucleic acid (tRNA), bound to eukaryotic initiation factor 2 (eIF2). Together, they all form the 43S pre-initiation complex (PIC). Binding of eIF3 and eIF4F positions the PIC at the 5' terminus of the mRNA molecule, forming the recruitment complex for final binding of the large 60S ribosomal subunit, initiating translation (Myasnikov *et al.*, 2009; Sonenberg and Hinnebusch, 2009b; Voigts-Hoffmann, Klinge and Ban, 2012; Ali *et*

al., 2017). In summary, the binding of eIF4F at the m^7 5' cap brings the ribosome to the 5' end of the mRNA strand, from where it starts scanning to find a start codon, all summarised in Figure 1 (Kong and Lasko, 2012).

Figure 1: Cap-dependent translation initiation, taken from Kong & Lasko, 2012

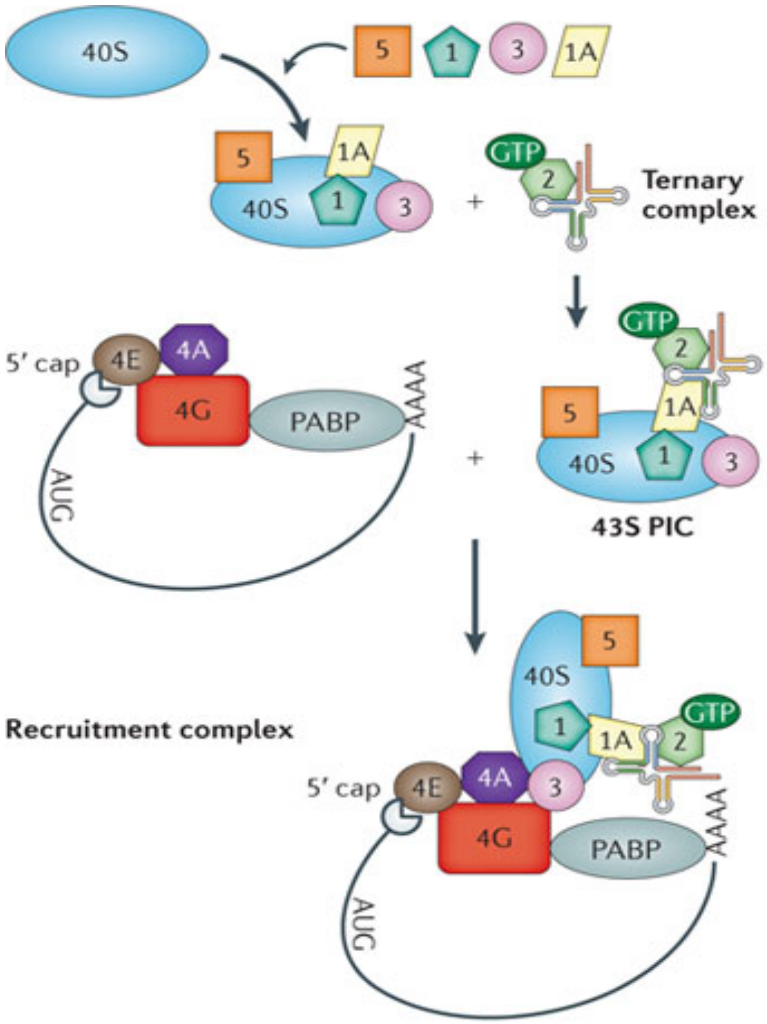


Figure 1 Legend: Canonical/Cap-dependent translation Initiation (Kong & Lasko, 2012)

Eukaryotic initiation factors (eIFs) help recruit ribosomal subunits to the m^7G 5' cap of an mRNA strand. eIF4E (brown) binds the cap directly and recruits factors eIF4G (red) and eIF4A (purple) to form a complex known as eIF4F. eIF4G (red) acts as a scaffold protein, that also binds poly-A binding protein (pAbp) (grey) which stabilises the mRNA molecule. eIF5 (orange), eIF1 (dark green), eIF3 (pink) and eIF1A

(yellow) bind the 40S ribosomal subunit (blue), together with the ternary complex, bound to activated eIF2 (light green). Altogether they form the 43S pre-initiation complex (PIC). eIF3 binds eIF4F, positioning the ribosomal subunit at the 5' end of the mRNA strand, where it begins to scan the molecule and initiates translation (Kong & Lasko, 2012).

1.2.2 Non-canonical/cap-independent translation initiation

Although the majority of mRNA is translated via a canonical, cap-dependent mechanism, eukaryotic cells have evolved at least 5 alternative initiation mechanisms in order to regulate gene expression in cases such as cell stress when energy is in demand. Approximately 10-15% of mRNAs are translated using alternative methods (Spriggs *et al.*, 2008). One primary alternative mechanism involves specific mRNA structures known as Internal ribosomal entry sites (IRES). These can recruit the 40S subunit independently of most cap-dependent factors. IRES-associated translation has been implicated with canonical factors such as eIF3 and eIF2, as well as IRES trans-activating factors (ITAFs). ITAFs aid in scaffolding other factors and recruiting ribosomal subunits (Sonenberg and Hinnebusch, 2007; Komar and Hatzoglou, 2011a; Mitchell and Parker, 2015). Other alternative mechanisms, including N6-methyladenosine (m^6A) modifications, which have been shown to stimulate translation in cases of both capped and uncapped mRNAs (Meyer *et al.*, 2015; Mitchell and Parker, 2015). 5'UTR M^6A modification of mRNA and YTHDF1-bound mRNAs promote the recruitment of ribosomal subunits and initiate translation independently of cap-dependent translation factors (Meyer *et al.*, 2015; Mitchell and Parker, 2015). Other initiation mechanisms include ribosome shunting and repeat-associated non-AUG (RAN) translation (Mitchell and Parker, 2015). Some of these methods still rely on eIF3 to recruit the pre-initiation complex and commence translation (Komar and Hatzoglou, 2011a; Mitchell and Parker, 2015). Yoffe et al. investigated cap-independent initiation in the context of embryonic stem cells. They knocked down death-associated protein 5 (DAP5) in human embryonic stem cells (hESCs), a factor which mediates Internal ribosomal entry site (IRES)-dependent translation. Knocking down this factor promoted the expression of self-renewal genes and delayed the expression of those associated with differentiation (Yoffe *et al.*, 2016). Alternative initiation mechanisms are summarised in Figure 2 (Mitchell and Parker, 2015). This research drew attention to alternative initiation mechanisms in the context of stem cell fate.

Figure 2: Cap-independent translation initiation methods, taken from Mitchell & Parker, 2015

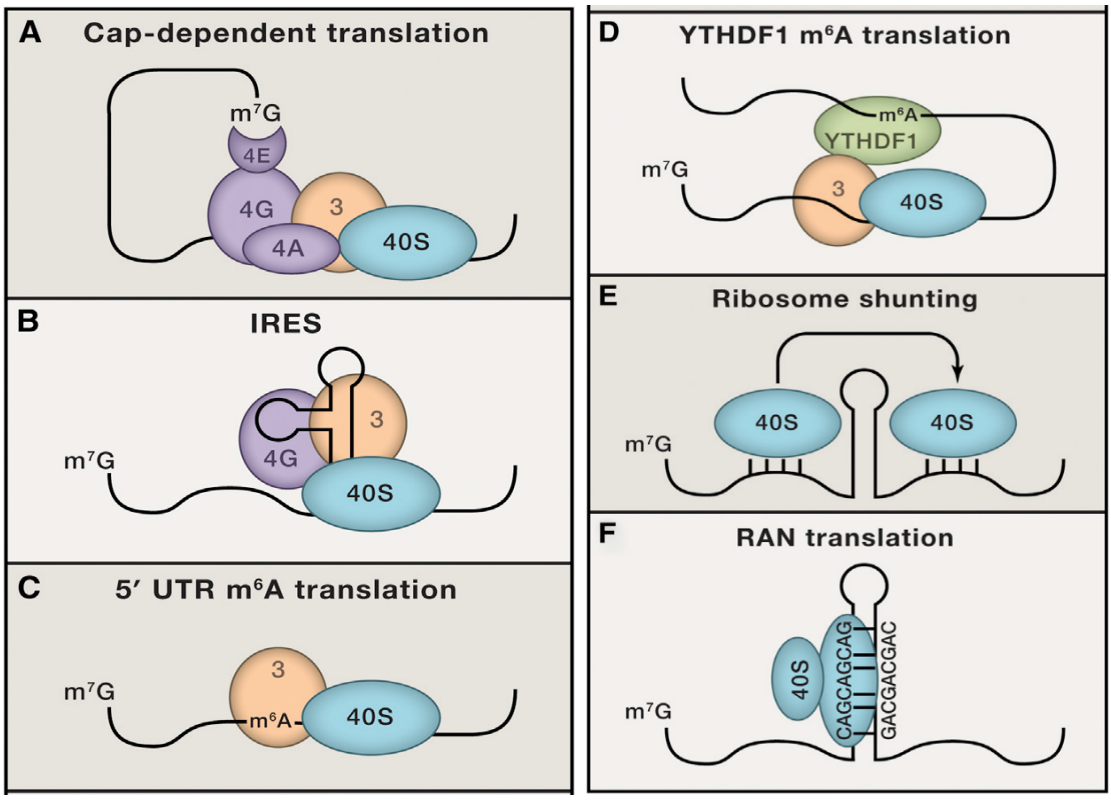


Figure 2 Legend: Cap-independent translation initiation, (Mitchell & Parker, 2015)

Alternative translation initiation methods. (A) Represents cap-dependent initiation of translation. eIF4F complex (purple) binds the m^7G 5' cap and recruits the 40S ribosomal subunit (blue) through eIF3 (orange) (Mitchell & Parker, 2015). (B) Represents internal Ribosomal Entry Site (IRES)-associated translation initiation. Certain mRNAs contain structures known as IRES, which can recruit ribosomal subunits independently of an m^7G 5'cap. The 40S subunit (blue) can either bind directly or indirectly through initiation factors, such as eIF4G (purple) or eIF3 (orange) (Mitchell & Parker, 2015). (C) Represents 5'UTR m^6A-mediated translation initiation. M^6A modifications recruit ribosomal subunits (blue) through recruitment of eIF3, initiating translation (Mitchell & Parker, 2015). (D) Represents YTHDF1-mediated translation initiation, where m^6A modifications at the 3'UTR of mRNA recruit YTHDF1 (green) and initiated translation with the help of eIF3 (orange) (Mitchell & Parker, 2015). (E) Represents ribosome shunting. The 40S ribosomal

subunit translocates (shunts) from the m^7G 5' cap to other start codons, where translation is initiated. Translocation is achieved through Ribosomal RNA base pairing with mRNA (Mitchell & Parker, 2015). (F) Represents repeat-associated non-AUG (RAN) translation initiation, where initiation occurs due to disease-associated CAG repeats (Mitchell & Parker, 2015).

1.3 The *Drosophila melanogaster* Model

1.3.1 The benefits of investigating a "fruit fly"

Due to the high redundancy of human genes and ethical constraints, it is difficult to study the genetics of humans. *Drosophila melanogaster* (dm) provides a simplified model for genetic experiments, with a small genome and low redundancy. One of the primary benefits includes a 75% chance of finding a homolog for disease-causing genes in humans (Roote and Prokop, 2013). The development of the fruit fly is 10 days from egg to adult, which enables genetic manipulation by cross-breeding to be very cost and time effective. A female fly lays up to 100 eggs per day, which allows large sample sizes to be generated for experiments. The *Drosophila* genome is condensed into a diploid, 4-chromosome genome. It has been sequenced and is highly accessible, with considerable availability of mutants to use in experiments (Roote and Prokop, 2013). This provides a model, which is easy to manipulate, giving rise to opportunities to identify proteins and signalling pathways that may play a role in higher organisms.

1.3.2 The *Drosophila* testis niche

The *Drosophila* testis is a highly tractable model to study stem cell behaviour due to certain underlying advantages. These include a short generation time, availability of genetic tools and unparalleled opportunities to observe and identify cells. Both stem cells and their niche can be identified by position and morphology, and markers for each cell fate are readily available (Losick *et al.*, 2011). Stem cells require a specific environment, called a niche, to provide them with adequate signals and support to maintain self-renewal. In the *Drosophila* testis, the niche is a group of 12 cells, called the hub (Matunis, Stine and de Cuevas, 2012). Hub cells are post-mitotic, which adhere to the testis apex and protrude into the testis lumen to bind and provide self-

renewal signals to adjacent cells (Matunis, Stine and de Cuevas, 2012). The hub supports two stem cell populations; somatic cyst stem cells (CySCs) and germline stem cells (GSCs) (Hardy *et al.*, 1979; Leatherman and Dinardo, 2008; Zoller and Schulz, 2012). Approximately 9-14 GSCs mitotically divide to give rise to daughter cells known as gonialblasts. Gonialblasts undergo 4 rounds of mitosis with incomplete cytokinesis to form cell cysts. The 16 cell cysts then undergo meiosis to form spermatids (Yamashita, Jones and Fuller, 2003; Sheng and Matunis, 2011; Matunis, Stine and de Cuevas, 2012). GSCs are in contact with the hub and once displaced from the niche become differentiating gonialblasts (Matunis, Stine and de Cuevas, 2012). Around 13 CySCs give rise to post-mitotic, somatic cyst cells which envelop developing gonialblasts and regulate their differentiation process (Hardy *et al.*, 1979; Zoller and Schulz, 2012). CySCs express a number of self-renewal factors, one the best characterised being Zinc finger homeodomain-containing protein (Zfh1). Zfh1 is required for CySC self-renewal and its expression is lost as CySCs differentiate into cyst cells (Leatherman and Dinardo, 2008). By contrast, differentiating cyst cells begin expressing the nuclear phosphatase Eyes absent (Eya) (Fabrizio, Boyle and DiNardo, 2003; Leatherman and Dinardo, 2008). The architecture of the testis is summarised in Figure 3 (Amoyel and Bach, 2012). The focus of the present project is on CySCs as there is a well-established understanding of signals that promote self-renewal and differentiation in the cyst lineage.

Figure 3: The Drosophila testis niche, taken from Amoyel & Bach, 2012

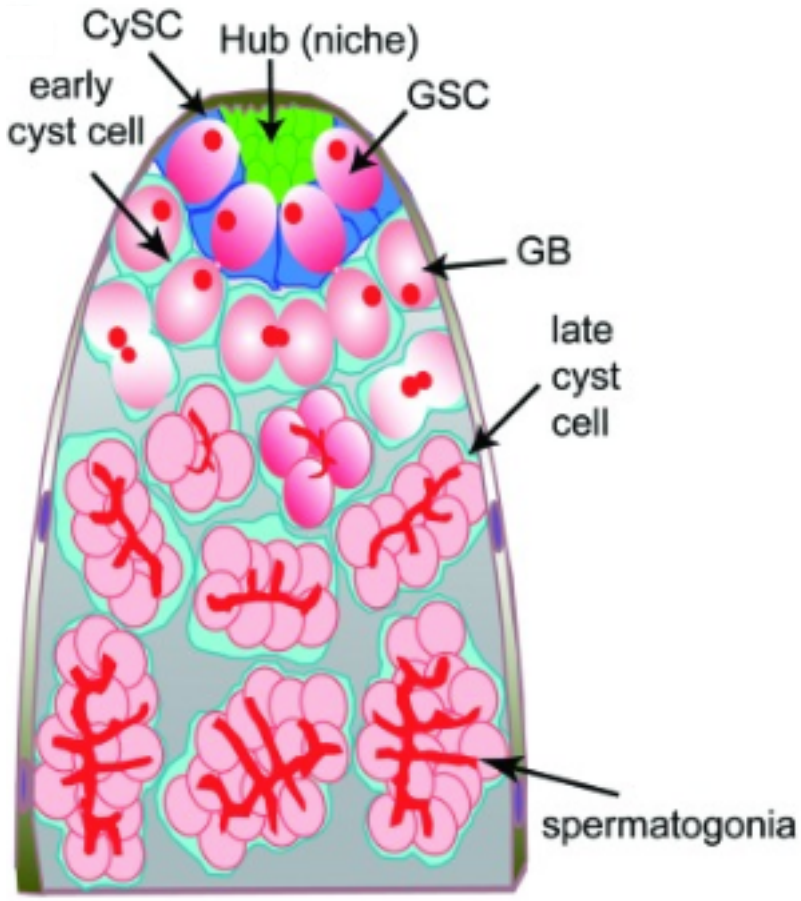


Figure 3 Legend: The Drosophila testis model, (Amoyel & Bach, 2012)

The Drosophila testis niche consists of approx. 12 hub cells (green) that provide self-renewal signals and support two stem cell populations; cyst stem cells (CySCs) (dark blue) and germline stem cells (GSCs)(dark pink). CySC divide asymmetrically, and give rise to post-mitotic cyst cells (light blue). GSCs divide and give rise to gonialblasts (GB), which later generate spermatogonia (light pink). Cyst cells envelope developing germ cells and aid in their progression (Amoyel & Bach , 2012).

1.3.3 Niche self-renewal Signals

The balance of self-renewal and differentiation of stem cells maintains tissue homeostasis. These fates are regulated by a series of signaling pathways that act on stem cells to induce specific behaviours. Hub cells produce the ligand for the Janus

kinase-signal transducer and activator of transcription (JAK/STAT) signalling pathway, called Unpaired (Upd). Upd activates JAK/STAT transduction in CySCs and GSCs and promotes CySC self-renewal as well as GSC adhesion to the hub (Tulina and Matunis, 2001; Leatherman and Dinardo, 2008; Issigonis *et al.*, 2009; Leatherman and DiNardo, 2010). The niche supports each lineage, within which cells compete for niche occupancy, a process referred to as neutral competition (Amoyel and Bach, 2014; Amoyel, Anderson, *et al.*, 2016). Hedgehog (Hh) and Hippo (Hpo) pathways induce bias in neutral competition within the CySC lineage, which can also lead to displacement of GSCs, allowing CySCs to colonise the niche through accelerated proliferation (Amoyel and Bach, 2014). CySCs require Hedgehog (Hh) signaling to maintain self-renewal in addition to JAK/STAT activation (Michel *et al.*, 2012). However, Hh and JAK/STAT have been shown to act independently of one another in their function. The Hh ligand is secreted by hub cells to promote self-renewal of CySCs, but not GSCs (Amoyel *et al.*, 2013). The niche also produces bone morphogenetic proteins (BMPs), such as Decapentaplegic (Dpp), which maintain self-renewal in GSCs (Shivdasani and Ingham, 2003; Kawase *et al.*, 2004; Zheng *et al.*, 2011; R. N. Wang *et al.*, 2014). Hub cells express Slit, a ligand that binds Roundabout 2 (Robo2) and allows CySCs to compete for a spot in the niche. The interaction regulates CySC adhesion through their effector Abelson tyrosine kinase (Abl) and JAK/STAT activation (Stine and Matunis, 2013). In summary, hub cells secrete several ligands, including; Upd, Hh, BMPs and others that maintain self-renewal in the niche. In addition, BMPs are also secreted by CySCs to promote self-renewal in GSCs, such that the niche for GSCs consists of both the hub and CySCs. Once stem cells exit the niche, their fate is in question due to the lack of self-renewal signals.

1.3.4 Regulation of differentiation in the niche

As stem cells divide, their daughter cells are pushed away from the niche. Losing access to the hub means that these cells experience a lack of self-renewal signals and therefore begin to differentiate. Daughter cells of CySCs that move away from the hub no longer receive Upd ligand and do not transduce JAK/STAT signal (Sinden *et al.*, 2012). This leads to their differentiation into cyst cells, as JAK/STAT has been shown to be necessary and sufficient to maintain CySC self-renewal, regulating the transcription of proteins induced during self-renewal such as Zfh1 (Leatherman and Dinardo, 2008). Loss of signals such as Upd defines differentiation as a “passive”

process regulated by access to the hub. Once outside the niche, differentiated cyst cells, envelope gonialblasts by extending their membranes around them (Schulz *et al.*, 2002). Later, occluding junctions form between cyst cell membranes to envelop germ cells, forming a permeability barrier, which isolates the niche from differentiating cells (Fairchild *et al.*, 2016). The barrier allows for signals from the niche to be restricted to neighbouring cells, promoting the differentiation of cells beyond the niche (Fairchild *et al.*, 2016). Recent work has shown that, in addition, there are signals that promote differentiation, suggesting that there is an active regulation of the differentiated cell fate. Epidermal growth factor (EGF) signaling plays an active role in promoting the division and differentiation of CySCs. The EGF receptor (EGFR) ligand Spitz (Spi) is processed and activated in the germline and stimulates cyst cells through EGFR (Schulz *et al.*, 2002; Sarkar *et al.*, 2007). EGF signaling has been shown to induce the expression of certain genes associated in cyst lineage differentiation, such as Eya (Salzer, Elias and Kumar, 2010). Recent research has also found a role for nutrient-sensitive PI3K/Tor pathway in promoting the differentiation of CySCs (Amoyel, Hillion, *et al.*, 2016). Overall, the regulation of differentiation is a recent observation, where regulation is mainly reduced to hub proximity and access to self-renewal factors, but a number of “active” mechanisms are now being identified.

1.4 The PI3K/Tor Pathway

1.4.1 Tor in differentiating CySCs

Research into the Phosphatidylinositol 3-kinase and target of rapamycin (PI3K/Tor) pathway has shown that CySC clones with hyperactivated PI3K/Tor activity differentiate and that knocking down Tor prevented differentiation. This suggests that Tor activity is necessary for differentiation, thus identifying an “active” role for this pathway in differentiation (Amoyel, Hillion, *et al.*, 2016). The PI3K/Tor pathway is sensitive to amino acid availability, growth factors and the energy status of the cell with downstream effectors including metabolism, cell growth and protein synthesis. Tor operates in 2 complexes; Target of rapamycin (Tor) acts in complexes Tor complex 1 (TORC1) and 2 (TORC2). TORC2 is less well understood but is known to regulate actin organization and endocytosis (Rispal *et al.*, 2015). In CySCs, differentiation is regulated by TORC1. Amoyel et al. have demonstrated that cyst cell differentiation is sensitive to Rapamycin, which only inhibits TORC1 (Takahara and

Maeda, 2012; Amoyel, Hillion, *et al.*, 2016). The group also knocked down Raptor, a component of TORC1, leading to a differentiation defect in cyst cells. These mimicked previous knockdowns of protein kinase B (Akt) and Tor (Amoyel, Hillion, *et al.*, 2016). Thus, TORC1 is required during differentiation in the cyst lineage, which leads to the question of what cellular process is affected downstream of TORC1 to promote differentiation.

1.4.2 Tor and cap-dependent translation

TORC1 regulates growth at least in part via its well-characterised targets: ribosomal protein S6 kinase (S6K) and eIF4E binding protein (4E-BP). TORC1 inhibits the activity of 4E-BP through phosphorylation. 4E-BP competes with eukaryotic initiation factor 4G (eIF4G) for the eIF4E binding site, and thereby prevents the assembly of the cap binding complex eIF4F, inhibiting translation. Phosphorylation of 4E-BP by TORC1 causes its dissociation from eIF4E, thus allowing the eIF4F complex to form, promoting the initiation of cap-dependent translation. S6K is involved in the regulation of protein synthesis by phosphorylating eukaryotic initiation factor 4B (eIF4B), which promotes its recruitment to eukaryotic initiation factor 4A (eIF4A), promoting translation initiation (Miron *et al.*, 2001; Showkat, Beigh and Andrabi, 2014).

Figure 4: The relationship between the Tor pathway and cap-dependent translation, adapted from Kong & Lasko, 2012

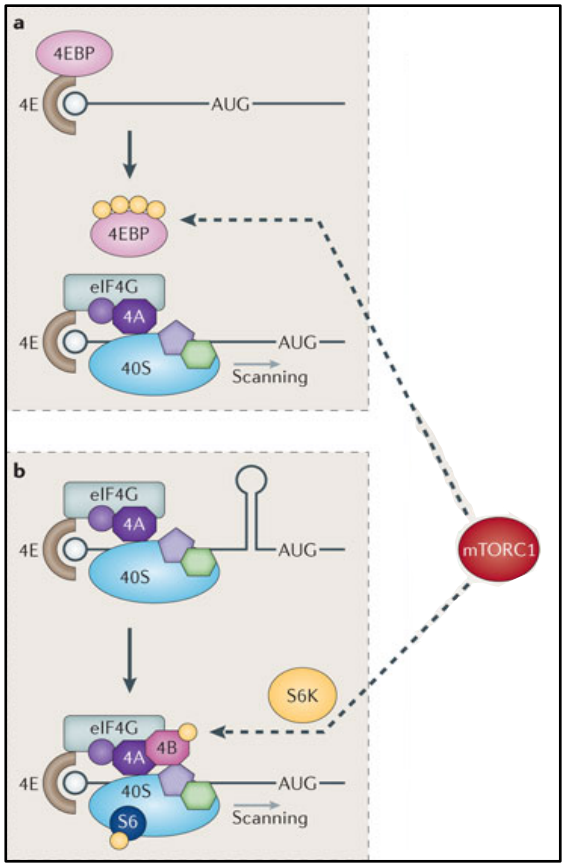


Figure 4 Legend: The relationship between the Tor pathway and cap-dependent translation, (Kong & Lasko, 2012)

Translation can be regulated downstream of the PI3K/Tor pathway through two main factors. (A) mTORC1(dark red) phosphorylates 4E-BP, inhibiting its ability to bind eIF4E (brown). eIF4G (dark green) can thus bind eIF4E (brown) allowing the eIF4F complex to form (multi-coloured) and translation to initiate. mTORC1 (dark red) also activates (B) S6K (yellow), which in turn phosphorylates eIF4B (pink-purple), promoting the recruitment of eIF4A (dark purple) and the formation of eIF4F (multi-coloured).

1.5 Aims and Objectives

Recent work in various stem cell models such as mouse HSCs and *Drosophila* GSCs has identified a potential regulatory role for translation in stem cell fate (Signer *et al.*, 2014; Sanchez *et al.*, 2016). In the *Drosophila* testis cyst lineage, PI3K/Tor signalling promotes differentiation (Amoyel, Hillion, *et al.*, 2016). It is expected that increasing PI3K/Tor signalling in differentiating cells should cause translation to increase as translation is one of the best-characterised outputs of this signalling pathway (Showkat, Beigh and Andrabi, 2014). Altogether, this poses the question as to whether translation could play a role in CySC differentiation and add to the growing list of processes that actively modulate this fate. It may be inferred from the literature that translation rates increase with increasing PI3K/Tor in differentiating cyst cells, matching current findings in HSCs and GSCs (Signer *et al.*, 2014; Sanchez *et al.*, 2016). However, preliminary data from the Amoyel lab have suggested that protein synthesis in fact decreases in the cyst lineage. O-propargyl-puromycin (OPP) protein synthesis assays (Liu *et al.*, 2012) have revealed that protein synthesis decreases in the cyst lineage during differentiation. These conflicting results draw attention to the role of translation in the cyst lineage of the *Drosophila* testis. The present work harnesses the powerful genetics of *Drosophila,* combining RNA interference (RNAi) silencing (Kavi *et al.*, 2005) techniques and Gal4/Gal80 transcriptional control methods (Suster *et al.*, 2004), to assess how translation is regulated during CySC differentiation. Here, I aim to measure translation rates using OPP assays, and to carry out a screen to identify how translation initiation factors affect cell-fate choices in the cyst lineage. My objectives also include preliminary characterisation and validation of some of the screen hits.

2.0 Material and Methods

2.1 Fly Stocks and Husbandry

Drosophila melanogaster (fruit flies) were kept and bred following standard practice (Roote and Prokop, 2013), in vials (*Drosophila* tubes 25x95 mm (PS), Dominique Dutcher) on food sourced on site. Most stocks were kept at 18°C and tipped onto fresh food every 4 weeks, a full list of stocks can be found at the end of each section. A list of all fly stocks used can be found in Table 1.

2.1.1 Protein Synthesis Assay of Control Testes

Our initial OPP investigations used unstaged, male Oregon R flies (Amoyel Stock) that were kept at room temperature (22°C).

2.1.2 Knockdown Screen

Crosses were organised to produce knockdown flies for specific cap-dependent and cap-independent translation initiation factors. Upstream activation sequence-RNAi (UAS-RNAi) construct lines were sourced from the Vienna or Bloomington Stock Centres. Each was crossed with *Gal4/Gal80* system virgins to produce offspring, which expressed RNAi under the control of the cyst lineage-specific promoter Traffic Jam (Tj). Coupling *Tj-Gal4* (Brand and Perrimon, 1993) expression with thermosensitive *Gal4* inhibitor $Gal80^{Ts}$ (McGuire, Mao and Davis, 2004), allowed me to regulate the expression of RNAis to adulthood.

It was necessary to control *Gal4* expression so that RNAi transgenes were only expressed in adulthood so to minimise any widespread effects of disrupted translation. All crosses were incubated at 18°C to allow *Gal4* function to be efficiently repressed by $Gal80^{ts}$, which is most effective at lower temperatures. Crosses were inspected daily and tipped twice a week as I collected F1 males of the desired genotype. Stocks were also kept at 18°C, with the exception of *w;Tj-Gal4/Cyo;tub-*$Gal80^{Ts}$*/TM6b*, which was maintained at room temperature (22°C), with virgins collected up to 3 times a day. Virgins were identified based on paler colouring and a black mark on their underside indicating sexual immaturity. When selecting flies, they were tipped onto a CO_2 pad (Diffuser Pad, Genese Scientific) and inspected under a

dissection microscope (MZ6, Leica). Once F1 male flies of the desired genotype were collected from each cross, I transferred them to a 29°C incubator for 10 days to promote the function of *Gal4* as $Gal80^{Ts}$ is inactive at this temperature. 10 days provided sufficient time for the expression of RNAis and for an effect to take place. After 10 days males were dissected and the testes were immunostained (Amoyel, Anderson and Bach, 2014)., mounted on slides, and scanned using a Leica SP8 confocal microscope.

2.1.3 MARCM Experiments

I crossed *y,w,hs-flp*^{122}*,tub-Gal4,UAS-nls-GFP/FM7; tub-Gal80, FRT*^{40A} virgins with males of the genotype ;*eIF4A*^{1013}*, FRT*^{40A} or ;*eIF4A*^{1006}*, FRT*^{40A} or *FRT*^{40A} control. Using the Mosaic analysis with a repressible marker (MARCM) system, I was able to generate positively labelled homozygous mutant cells, which were either homozygous for the alleles of eIF4A or for a control chromosome. This used flp-FRT mediated mitotic recombination. Using *UAS-Gal4* and *Gal80* systems I labelled clones using UAS-GFP expression (Brand and Perrimon, 1993; Lee and Luo, 1999, 2001; McGuire, Mao and Davis, 2004). The crosses were incubated at 25°C, the optimal temperature for raising *Drosophila,* and collected from 3 times a week. eIF4A mutant clones were generated by MARCM technique after heat-shocking for 1 hour in a 37°C water bath (Lee and Luo, 1999, 2001). The males were then returned to 25°C incubation for 48 hours or 7 days before dissecting and staining using standard protocol.

2.1.4 Knockdown Protein synthesis Assay

I repeated the crosses of eIF4E1, eIF4A, eIF4G, eIF3a and eIF2alpha RNAis from the screen and submitted them to the exact same conditions. I incubated selected males at 29°C for 2 days for each genotype. This was so that I could investigate the translation rate in CySCs, where the RNAi had taken effect but had not yet caused a new phenotype. After 2 days I dissected and stained testes using an OPP click-IT reaction protocol.

2.1.5 Rapamycin Experiments

I repeated the crosses of the screen eIF4F complex hits; eIF4E1, eIF4A and eIF4G RNAis. The crosses were maintained and set up in the exact same conditions as described above for my screen. However, males of the desired genotype were incubated at 29°C on both control and Rapamycin-containing food for 10 days. I obtained Rapamycin from LC Laboratories and prepared the food by adding 100µl of a 4mM stock solution in ethanol to each food vial and letting it air dry. Rapamycin treatment was not negatively controlled with a vehicle treatment, i.e. ethanol. Selected males were transferred to fresh Rapamycin-containing food every 2 days over a 10-day period before being dissected and immunostained (Amoyel et al., 2016).

2.1.6 p4E-BP Staining

I repeated the same crosses for eIF4E1, eIF4A and eIF4G RNAis and submitted them to the same conditions as for the screen. Collected F1 males, however, were transferred to 29°C for 2 days instead of 10 days so that I was able to measure p4E-BP levels in CySCs (CySCs were not present after 10-day incubation at 29°C). The flies were then dissected and stained using a phospho-epitope staining protocol (Schultz et al., 2002).

2.1.7 Stat92E and pJnk Investigation

I repeated the same crosses as the screen for eIF3a and eIF2alpha RNAis and submitted them to the same conditions. Collected F1 males were transferred to 29°C for 10 days, dissected and stained using standard and phospho-epitope staining protocols for Stat92E and pJnk respectively.

Table 1: Fly stocks and genotypes

Type	Factor	Source	Stock Number	Genotype
Control	n/a	Amoyel Lab	n/a	*Tj-Gal4; tub-Gal80^{Ts}*
RNAi	eIF1	Vienna	v29216	*w[1118]; P{GD14502}v29216*
RNAi	eIFIA	Vienna	v26022	*w[1118]; P{GD10618}v26022*
RNAi	eIF2alpha	Vienna	v7799	*w[1118]; P{GD1430}v7799*
RNAi	eIF2gamma	Vienna	v39377	*w[1118]; P{GD15258}v39377/CyO*
RNAi	eIF2Balpha	Vienna	v40321	*w[1118] P{GD10611}v40321*
RNAi	eIF3a	Vienna	v28140	*w[1118];; P{GD12569}v40321*
RNAi	eIF3b	Vienna	v107829	*P{KK107829}VIE-260B,VIE-40D*
RNAi	eIF4A	Vienna	v42202	*w[1118]; P{GD14111}v42202*
RNAi	eIF4B	Vienna	v31364	*w[1118]; P{GD7103}v31364*
RNAi	eIF4E1	Vienna	v7800	*w[1118]; P{GD1432}v7800*
RNAi	eIF4EHP	Vienna	v38399	*w[1118]; P{GD7071}v38399*
RNAi	eIF4E3	Vienna	v34210	*w[1118]; P{GD10616}v34210*
RNAi	eIF4E4	Vienna	v107595	*P{KK105485}VIE-260B*
RNAi	eIF4E6	Vienna	v17580	*w[1118]; P{GD8319}v17580*
RNAi	eIF4G	Vienna	v17003	*w[1118]; P{GD7098}v17580*
RNAi	eIF4H1	Vienna	v34301	*w[1118]; P{GD10708}v34301*
RNAi	eIF4H2	Vienna	v102825	*P{KK103972}VIE-260B*
RNAi	pAbp	Vienna	v22007	*w[1118]; P{GD11542}v22007*
RNAi	GlyRS	Vienna	v44606	*w[1118]; P{GD12037}v44606*
RNAi	eIF6	Vienna	v108094	*P{KK101259}VIE-260B*
RNAi	CG7483	Vienna	v108580	*P{KK101462}VIE-260B*
RNAi	eIF2D	Bloomington	33995	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00958}attP2*
RNAi	DENR	Vienna	v28105	*w[1118]; P{GD12540}v28105*
RNAi	squid	Vienna	v32395	*w[1118]; P{GD8593}v32395*
RNAi	heph	Vienna	v33735	*w[1118]; P{GD10126}v33735*
RNAi	Unr	Vienna	v49498	*w[1118] P{GD17178}v49498*
RNAi	syp	Vienna	v33011	*w[1118]; P{GD9477}v33011*
RNAi	Hrb87F	Vienna	v51759	*w[1118]; P{GD7818}v51759*
RNAi	La	Vienna	v2988	*w[1118]; P{GD1476}v2988*
RNAi	Larp	Bloomington	41835	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL01263}attP2/TM3, Sb[1]*
RNAi	YTHDF	Bloomington	55151	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03791}attP40*

RNAi	YTHDC1	Bloomington	34627	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01302}attP2*
RNAi	SAM-S	Bloomington	29415	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03351}attP2/TM 3, Sb[1]*
Marcm	n/a	F.Schweisgut h	n/a	*y,w,hs-flp^{122},tub-Gal4,UAS-nls-GFP/FM7;tub-Gal80, FRT^{40A}*
Marcm	n/a	Amoyel Lab	n/a	*w;FRT40A*
Marcm	n/a	Dr. Ting Xie	n/a	*w;eIF4A^{1013}, FRT^{40A}*
Marcm	n/a	Dr. Ting Xie	n/a	*w;eIF4A^{1006}, FRT^{40A}*

Table 1 Legend: Fly stocks and genotypes

This table outlines all fly stocks used in my experiments. Column 4 outlines the source of each stocks, most of which were sourced from the Vienna Drosophila Resource Centre (VDRC or Vienna), however some were sourced from Bloomington Drosophila Stock Centre (Bloomington) and other labs. The genotypes are stated as they are on the respective source websites and contain details of the transgenic construct used (GD/KK/TRiP).

2.2 Immunohistochemistry

The following primary antibodies were used: rabbit anti-Zfh1 (gift of C. Desplan, NYU, NY, USA), 1:500; guinea pig anti-Traffic jam (gift of D. Godt, University of Toronto, Ontario, Canada), 1:5000; mouse anti-Armadillo (DSHB), 1:20, goat anti-Vasa (Santa Cruz, 26877), 1:400; mouse anti-Fas3 (DSHB), 1:20; mouse anti-Eya (DSHB), 1:20. Mouse anti-Dlg (DSHB), 1:20, chicken anti-GFP (Aves laboratories) 1:500, rabbit anti-Stat92E (Flaherty et al 2010), 1:500, rabbit anti-pJnk (gift of E. Piddini lab), 1:500, rabbit anti-p4E-BP (Cell Signaling Technologies).

2.2.1 Standard Immunostaining

When staining for my RNAi screen, MARCM and Rapamycin investigations I followed a standard protocol as previously described (Amoyel, Anderson and Bach, 2014). I

dissected flies in phosphate-buffered saline (PBS), then fixed in 4% paraformaldehyde (PFA) for 15 mins. The samples were then washed in PBS-Triton 0.5% for 30 mins, twice, then blocked in PBS-Triton 0.2% Triton with Bovine Serum Albumin (PBTB) for one hour and finally incubated overnight in a primary antibody mixture at 4°C in PBTB. The day after I washed the samples in PBTB twice for 30 mins, followed by placing them in secondary antibody mixture in PBTB for 2 hours. This was followed by washes in PBS-Triton 0.2% for 30 mins, twice. A similar protocol was followed for our investigation of Stat92E levels in eIF3/2 RNAi testes, but I adjusted the protocol by incubating in primary antibodies at room temperature overnight to achieve better staining results. All testes were mounted by pulling out the insides of the fly abdomen and separating the testes from the other organs using forceps. These were mounted onto slides using Vectashield (Vector labs), which I then analysed under a Leica SP8 confocal microscope.

2.2.2 Phospho-epitope Staining

When I investigated levels of p4E-BP and pJnk I followed a phospho-epitope staining protocol previously described in Schulz et al., 2002. The protocol resembled that of a standard immunostain, however dissections were carried out in 10 mM Tris-HCl, pH 6.8, 180 mM KCl, 50 mM NaF, 10 mM $NaVO_4$ and 10 mM β-glycerophosphate. Samples were fixed and stained as in the standard staining protocol (see above).

2.2.3 O-Propargyl-Puromycin

Staining was achieved using Click-iT Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit (Thermofisher) and following the protocol described in the kit manual (Thermofisher). I adjusted the protocol slightly, by performing an immunostaining as described above and in (Amoyel, Anderson and Bach, 2014), before carrying out the Click-iT reaction (Liu *et al.*, 2012). Instead of dissecting in PBS, I dissected flies in Shields and Sang M3 insect medium to maintain the tissue, then incubated in OPP reagent for 1 hour at room temperature, with shaking before fixing. I prepared a reaction cocktail containing 880uL 1X reaction buffer, 20uL copper protectant, 100uL 1X additive and 2.5 uL Alexa fluor picolyl azide. This was added to the samples after incubation in secondary antibodies, for 30 mins. After the colour reaction, samples were washed in PBT 0.2% for 15 mins, twice, before mounting.

2.3 Analysis

Each sample was visualised using Leica SP8 confocal microscope. Testing significance for datasets first used a visual normality test, generated by looking at and plotting the data. This was followed by an unpaired t-test, assuming a two-tailed distribution and a two-sample unequal variance. All statistics calculations were made using Microsoft Excel.

2.3.1 Protein Synthesis Assay of Control Testes

I identified Tj-positive CySCs with the help of anti-Armadillo antibody, which labels cell outlines. CySCs were defined as Tj-positive nuclei one cell diameter from the hub, that had membrane extensions that contacted the hub. Differentiating daughters made no contact with the hub and were found outside the first two rings of cells. Once identified, I measured OPP incorporation through ImageJ measurement Mean Gray Value in the green (1^{st}) channel. I measured this for both CySCs and immediate daughter cells. I calculated the mean of each set of data and the significance using an unpaired t-test, giving a p value < 0.04.

2.3.2 Knockdown Screen

Each RNAi knockdown phenotype was analysed according to the presence/absence of Zfh1-positive cells, whether Zfh1-positive cells were identified outside the niche, the presence/absence of Eya-positive cells, the presence/absence of Fas3-positive hub and the accumulation of Topro staining. In normal GSC differentiation Topro staining accumulated around the niche. Where I found abnormal GSC differentiation, Topro staining either accumulated throughout the testis or not at all. 1/0 was awarded for presence/absence of cells/hub and 1/0 was awarded to normal/abnormal GSC differentiation. I then generated an average for each category and converted it to a percentage.

2.3.3 MARCM Experiments

The data were distributed according to the age of testes post-clone induction. I assessed each phenotype separately, using a scoring method. A clone was detectable through GFP expression and awarded a 1/0 for its presence/absence in each testis. I scored for the presence of CySC-, cyst cell-, GSC- and gonialblast-clones. An average was then generated for each category and converted to a percentage, enabling me to assess phenotypes.

2.3.4 Knockdown Protein Synthesis Assay

The data were analysed in the same way as the initial protein synthesis assay, however I also measured the translation rate in GSCs for each knockdown. I therefore compared Mean Gray Value (OPP incorporation) of CySCs and differentiating cyst cells and normalised CySC translation rates to GSC results. This enabled me to account for a bad stain and validated my results. For both sets of data I used an unpaired t-test to assess significance.

2.3.5 Rapamycin Experiments

Here I used the same scoring method as in the screen to determine differences between phenotypes and whether they were rescued.

2.3.6 p4E-BP Staining

When staining p4E-BP in RNAis contructs, I qualitatively assessed whether there was a change in p4E-BP levels compared to my control stains. The control showed a band of p4E-BP staining in the 2nd and 3rd rows of cells outside the hub, as previously described in Amoyel et al., 2016. A 1 was awarded to the phenotype I observed, i.e. increase, decrease or control p4E-BP phenotype, and a 0 if a phenotype was not

observed. The scores were averaged and converted to percentages so one could see how often specific phenotypes were recorded.

When staining p4E-BP in *eIF4A*^{1013} mutant clones I used Mean Gray Area measurement to determine staining intensity in the clones. I measured the intensity of the p4E-BP stain in CySC clones compared to control CySCs from the same testes. I analysed control testes in the same way to compare differences. I performed an unpaired t-test on the results to assess significance.

2.3.7 Stat92E and pJnk Investigations

Here, I qualitatively assessed whether there was a change in Stat92E/pJnk level compared to control stains. I awarded a 1 to the phenotype observed, i.e. ectopic/control Stat92E or increase/decrease/control pJnk phenotype, and a 0 to what was not observed. The scores were averaged and converted to percentages so one could see how often I observed a phenotype.

3.0 Results

3.1 Control O-propargyl-puromycin Assay

3.1.1 Translation rates are higher in CySCs compared to their differentiating daughters

Firstly, I sought to ascertain whether translation changed in the cyst cell linage during differentiation. Preliminary results from the Amoyel lab suggested that CySCs had higher translation rates than their daughters but this had not yet been quantified. I investigated the translation rates in CySCs and their immediate, differentiating daughters, by measuring protein synthesis using a O-propargyl-puromycin (OPP) Click-iT reaction assay (Liu *et al.*, 2012). The assay was conducted *ex vivo*, in Oregon R *Drosophila* testes, which is a standard control strain. This protein synthesis assay allows one to measure the rate of translation by incorporating a synthetic, modified puromycin compound into nascent polypeptides. OPP is detected after fixation and immunostaining by a covalent addition of a fluorescent azide using click chemistry. The more OPP is incorporated, the higher the rate of protein synthesis. I labelled CySCs and daughter cells using an antibody against Traffic Jam (Tj), and cell outlines and the hub using an antibody against armadillo (Arm) (see Fig. 5A-A’’’) (Loureiro and Peifer, 1998; Wingert and DiNardo, 2015). The majority of the hub’s surface area is in contact with GSCs (see Fig. 5A, 5A’’), whereas CySCs contact the hub with small membrane projections that can be found between GSCs. Therefore, CySC nuclei are located in a second row behind GSCs and the hub. CySCs (green arrows) were identified firstly using Tj (see Fig. 5A, 5A’) and secondly based on Arm (see Fig. 5A’’’) staining a membrane projection in contact with the hub (also stained with Arm). Differentiating daughter cells (see Fig 5A, 5A’, red arrows) were also identified using Tj (see Fig. 5A, 5A’) staining and due to a lack of contact with the hub (see Fig. 5A’’’). OPP incorporation was determined by measuring the Mean Gray Value of individual cells in ImageJ. 83% of CySCs (38/45) showed significantly higher rates of OPP incorporation ($p<0.04$, Student’s T-test, CySCs vs daughters) (see Fig. 5A’’, Fig. 5B) compared to their differentiating daughters (see Fig. 5A’’, Fig. 5B). These results imply that changes in overall protein synthesis levels accompany cell fate changes in the somatic lineage in the testis. The result that stem cells have higher translation levels than differentiating cells is unexpected, as previously published work showed that Tor activity is high in the differentiating daughter cells of CySCs (Amoyel, Hillion, *et al.*, 2016). Since Tor is known to induce translation (Miron

et al., 2001), I would have expected that differentiating cells with high Tor activity also had higher translation rates than CySCs.

Figure 5: CySCs synthesise more protein than their differentiating daughters

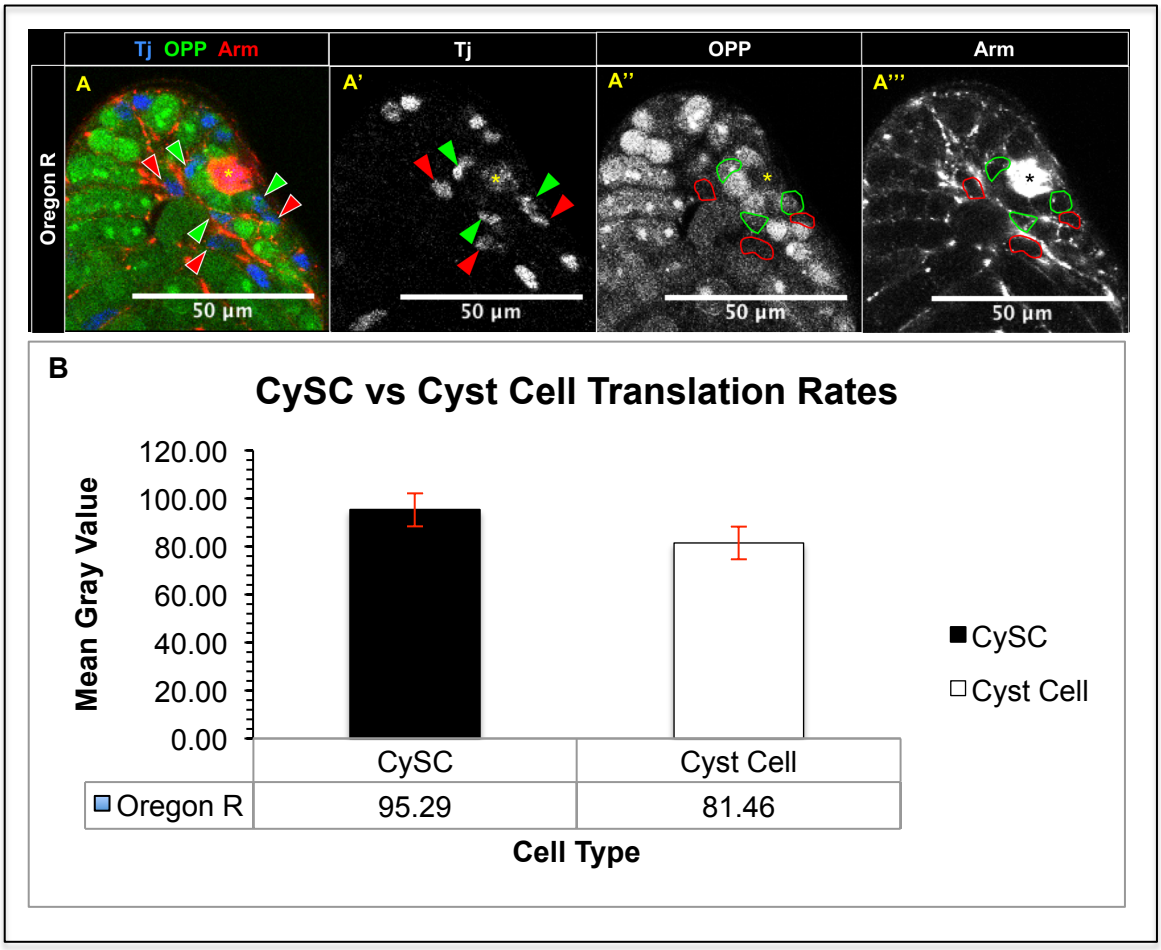


Figure 5 Legend: CySCs synthesise more protein than their differentiating daughters

(A-A’’’) Oregon R testis stained testis. Tj is expressed in the cyst lineage and so Tj (blue) was used to highlight CySCs (A-A’, green arrows) and cyst cells (A-A’, red arrows). The hub (asterisk) and cell membranes were stained using Vasa (red), with Tj-expressing CySCs contacting the hub (A’’’, green outline), whereas their Tj-expressing daughters did not (A’’’, red outline). OPP (green) incorporation was stronger in cells contacting the hub (A’’, green outline) compared to daughters, not contacting the hub (A’’, red outline). Images A through A’’’ have 50um scale bars.

(B) The bar chart displays the Mean Gray Value (MVG), used to determine the incorporation value of OPP, in individual CySCs (black) compared their daughter cells/cyst cells (white), with standard error bars (red). OPP incorporation was used to determine translation rates in different cells. Translation rates were significantly higher (P value < 0.04, Student’s T-test, CySC vs cyst cells) in CySCs (black) compared to their daughter cells/cyst cells (white).

3.2 RNAi Knockdown Screen: 5'cap-dependent Translation Initiation Factors

Since translation levels change during differentiation, I hypothesised that the regulation of translation might play a role in controlling cyst cell differentiation. Translation is regulated at the level of initiation, where assembly of the ribosomes at the mRNA is the limiting step. I speculated that different initiation factors may play a role in CySCs than in differentiating cyst cells, and thereby designed a screen to test whether different initiation factors were required for one fate or the other. The screen was conducted to investigate the role of translation in CySC fate control, in depth. I assessed the effect of knocking down several complex components, traditionally associated with cap-dependent translation initiation. This provided good coverage of the roles of these complexes in the context of CySC fate (Marygold, Attrill and Lasko, 2017). I knocked down initiation factors using the Gal4-UAS system (Brand and Perrimon, 1993) to drive an RNAi for each factor in the cyst lineage. I used *Tj-Gal4,* a cyst cell-specific driver expressed in both CySCs and differentiated cyst cells to drive RNAi expression. To restrict induction of the RNAi to adulthood and avoid developmental defects, I used a temperature-sensitive inhibitor of *Gal4, Gal80*^{ts}, which is active at 18°C but inactive at 29°C (McGuire, Mao and Davis, 2004; Suster *et al.*, 2004; Kavi *et al.*, 2005). First generation males were collected from each cross and kept at 29°C, the restrictive temperature for Gal80^{ts}, for 10 days. This allowed for knockdown of essential genes only in adult flies and circumvented any developmental defects that may have arisen if the RNAi transgene were expressed throughout development. Adult males were dissected and the testes immunostained using Zfh1 (to label CySCs), Fasciclin III (Fas3) (which labels the hub), Eya (to label differentiated cyst cells) and Topro-3-iodide (Topro) (a DNA stain to label all cell nuclei) antibodies. The testes were then mounted and imaged under a confocal microscope. The phenotypes were scored according to i) gain of CySCs, based on general increase in number and/or ectopic Zfh1-positive cells at a distance from the hub and ii) loss of CySCs, based on either a general decrease in number or a complete absence of Zfh1-positive cells (see Tables 2 & 3). I also scored for the presence of the hub and GSC differentiation based on Topro staining (see Table 3). The density of DNA-stained nuclei can be used to follow germ cell differentiation; high density reflects early stages while late stage germ cells have more dispersed nuclei, thus causing a less dense Topro staining. Unfortunately, in some cases it was not possible to score Topro differentiation due to some unsuccessful Topro stains. I scored such stains as n/a (see Table 3). As a control, I used flies carrying both *Tj-*

Gal4 and *Gal80^{ts}*, (*yw122;Tj-Gal4/+;Tub,Gal80^{Ts}/+)*, which were submitted to the same conditions as all knockdown flies. Knockdown phenotypes ranged in extremity and penetrance, from clear phenotypes with either no Zfh1-positive cells or multiple ectopic Zfh1-positive, to control-like phenotypes. A summary of the screen results can be found in Tables 2 and 3.

3.2.1 5' cap-dependent translation initiation factor eIF4E1 is required in CySC self-renewal whereas eIF4E6 is required in differentiating cyst cells

eIF4E binds the 5' cap and recruits eIF4A, along with eIF4G during the initiation of translation (Sonenberg and Hinnebusch 2009). In *Drosophila*, there are several eIF4E paralogues, most of which I looked at in the screen (Marygold, Attrill and Lasko, 2017). The previously described OPP results suggest that translation rates are significantly higher in CySCs than their differentiating daughters (see Fig. 5). Due to a higher need for translation in self-renewing cells, we expected all eIF4E knockdowns to result in a loss of CySC/Zfh1 phenotype. In testes in which eIF4E1 was knocked down (see Fig. 6B-B'''), I observed a complete depletion of Zfh1-positive CySCs (see Fig. 6B) in 53% (9/17) of testes, no hub (see Fig. 6B'') in 48% (8/17) of testes and Eya-positive cells in 100% of cases (see Table 3). I also observed a general reduction of Topro staining (see Fig. 6B''') in 95% (16/17) of testes (see Table 3), suggesting that GSCs were also lost non-autonomously. These results suggest that eIF4E1 may indeed play a role in the self-renewal of CySCs. Since Eya-positive cells were always observed, this suggests eIF4E1 is not required for cyst cell differentiation. Knocking down eIF4EHP produced an unclear phenotype. I observed Zfh1-positive cells (see Fig. 6C') and Eya-positive cells (see Fig. 6C'') in all (16/16) testes. I observed increased Topro staining in 12.5% (2/17) of cases (see Fig. 5C''', Table 3), implying that GSCs were unable to differentiate. I also observed a slight reduction in Zfh1-expressing cells (see Fig 6C'i), which implies a possible role in CySC self-renewal. eIF4E isoforms are characterised by their different binding affinities for the mRNA 5' cap with eIF4E3 binding with the highest affinity, however the respective knockdown phenotype (see Fig. 6D-D''') was not as strong as that of eIF4E1 (Zuberek *et al.*, 2016). I observed Zfh1-positive (see Fig. 6D') and Eya-positive (see Fig. 6D'') cells in 21/21 testes. Knocking down eIF4E3 resulted in a normal Topro stain (see Fig. 6D'''), implying no loss of GSCs. A slight reduction was observed in Zfh1-positive cell number (see Fig. 6D'i)) and hub size (see Fig 6C'', Fig.

6D''), suggesting a possible role in self-renewal. I observed similar results in the testes of eIF4E4 knockdowns (see Fig. 6E-E'''), where the phenotype resembled that of the control (see Fig. 6A) with no obvious differences. Unfortunately, I was unable to investigate GSC differentiation as my Topro staining was unsuccessful (see Fig. 6E'''). I observed a contrasting phenotype to eIF4E1, when knocking down eIF4E6. In 20% (3/15) testes I observed Zfh1-positive CySCs and ectopic Zfh1-positive cells away from the hub (see Fig. 6F-F'). In all testes (15/15), I observed an increased number of Zfh1-positive cells (see Fig. 6F, 6F') compared to control testes (see Fig. 6A, 6A') and increased Topro staining was recorded in 80% (12/15) testes, implying that GSCs were also unable to differentiate. Little is currently known of the exact function of eIF4E6 in the initiation of translation, however these results imply that eIF4E6 plays a role in promoting differentiation.

In summary, the results from eIF4E isoform knockdowns (see Fig. 6B-F''', Table 2) suggest that eIF4E paralogues promote CySC self-renewal, excluding eIF4E6. Knocking down eIF4E1 produces a strong phenotype (see Fig. 6B-B''') that suggests a role in promoting the self-renewal of CySCs (see Table 2). The role of eIF4EHP remains unclear, due to a slight reduction observed in CySCs in knockdown testes and impaired GSC differentiation. Knocking down eIF4E3 or eIF4E4 produced a control phenotype therefore one cannot conclude specific roles for these factors in CySC fate. However, RNA silencing is not always 100% effective with the chance of off-target effects (Qiu, Adema and Lane, 2005), therefore the exact role of these factors in CySCs is still debatable. Qualitative analysis of CySC number and hub size suggests a possible role in self-renewal (see Table 2). I also conclude that eIF4E isoforms do not share the same function in CySC fate, as eIF4E6 appears to play a role in differentiation (see Tables 2 & 3).

Figure 6 Legend: eIF4E knockdown phenotypes show impaired self-renewal, except eIF4E6 knockdown which shows impaired differentiation.

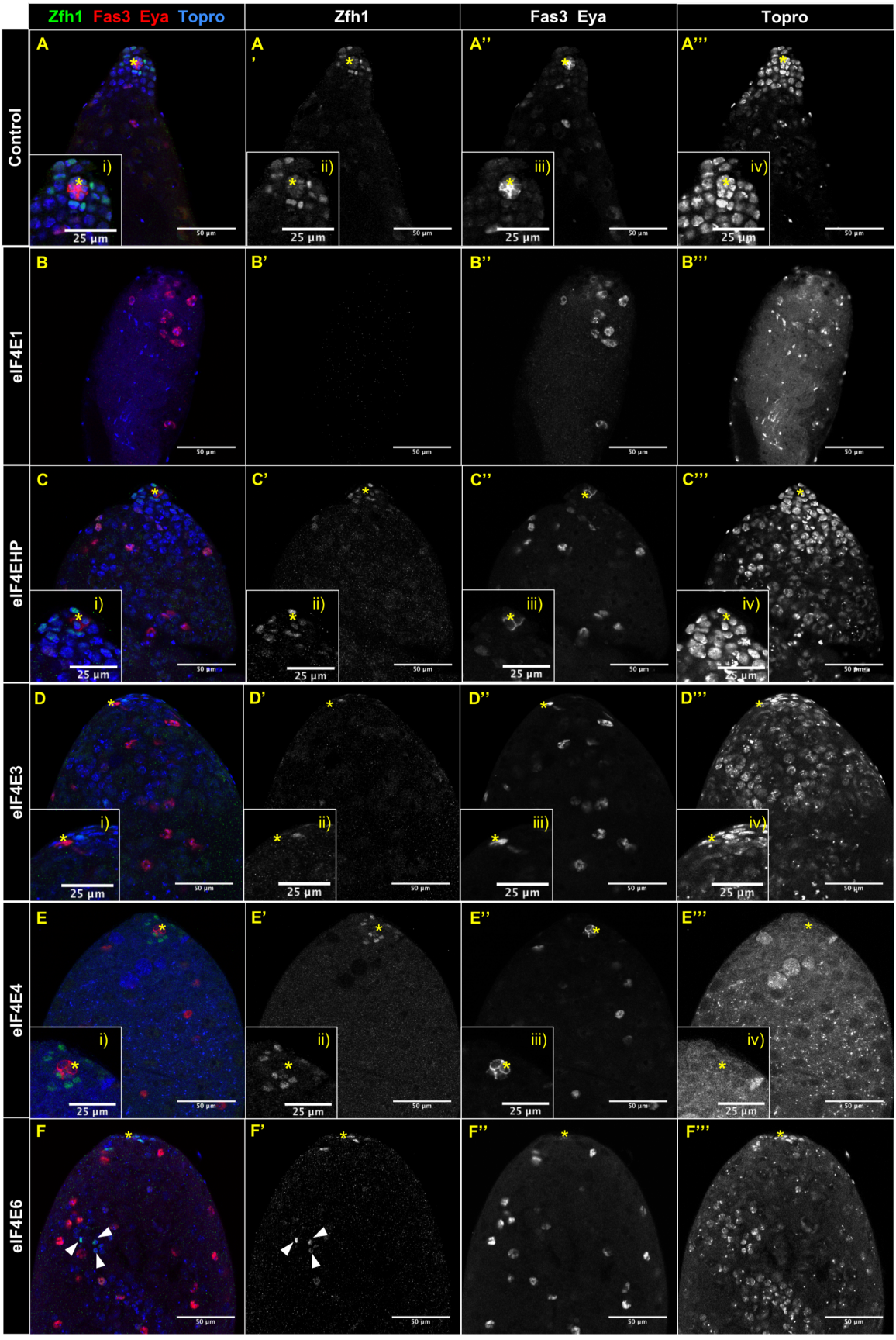


Figure 6 Legend: eIF4E1 and eIF4EHP knockdowns leads to loss of CySCs and eIF4E6 knockdown led to ectopic CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). Images A through to F''' have 50um scale bars displaying the testis apex and images i)-iv) of each genotype display a focus on the niche (asterisk) with a scale bar of 25um. Control testes (A-A''') had all cell types present (A, green), including Zfh1-expressing CySCs (A, i), A', ii), green), Eya-expressing cyst cells (A'', red) and a Fas-3 expressing hub (A'', asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A''', blue). Knocking down eIF4E1 (B-B''') resulted in complete depletion of Zfh1-expressing cells CySCs (B, B', green), with only Eya-expressing cyst cells remaining (B, B'', red) and abnormal GSC differentiation (B, B''', blue). This suggests a role for eIF4E1 in self-renewal. eIF4EHP knockdown (C-C''') resulted in a control cells being recorded (C', C''), however there were generally fewer Zfh1-expressing CySCs (C, i), C'', ii), green). In addition, GSC differentiation was inhibited, due to excess Topro staining (C, C''', blue). Knocking down eIF4E3 (D-D''') and eIF4E4 (E-E''') resulted in phenotypes very similar to the control, however in the eIF4E3 knockdown phenotypes there was a slight reduction in Zfh1-expressing CySC number (D, D', ii), green) around the Fas3-expressing hub (asterisk, red). Knocking down eIF4E6 (F-F''') resulted in a converse phenotype to other paralogue knockdowns. There was an increase in Zfh1-expressing CySCs (F, F', green) and multiple Zfh1-expressing CySCs outside the niche/hub (asterisk, red) (F, F', green, arrows), along with impaired GSC differentiation, due to unusual Topro staining (F'''). This suggests that eIF4E6 may play a role in cyst cell differentiation.

3.2.2 5’ cap-dependent translation initiation factors eIF4A and eIF4G promote self-renewal in CySCs

Next, I knocked down the other eIF4F components, eIF4A, eIF4B, eIF4G, eIFH1 and eIF4H2. eIF4A and eIF4G are recruited to the 5’ cap by eIF4E, and assemble eIF4F at the 5’ cap. Since eIF4E1 knock down resulted in loss of Zfh1-expressing CySCs, and since eIF4E is important for assembling the complex at the 5’ cap, I expected to observe the same phenotypes for these knockdowns (Sonenberg and Hinnebusch 2009). Knocking down eIF4A in the cyst cell lineage (Fig. 7B-B’’’) led to a complete loss of Zfh1-positive CySCs in 83% (14/17) of testes (Fig. 7B’) and a hub loss in 22% (4/17) (see Table 3) of testes. The only remaining cells were Eya-positive differentiated cyst cells (Fig. 7B’’) and increased Topro staining was present in all testes, implying that GSCs were unable to differentiate (see Fig. 7B’’’). The loss of Zfh1-positive cells suggests that eIF4A is required for CySC self-renewal. eIF4A is known to bind with eIF4E directly, and knocking down this factor mimics the phenotype of eIF4E1 loss (see Fig. 7B-B’’’), therefore suggesting that they could act in a complex to maintain CySC self-renewal. eIF4B interacts with eIF4A and binds the eIF3 complex when initiating translation. Knocking down eIF4B (see Fig. 7C-C’’’) caused no obvious differences from the control testes (see Fig. 7A-A’’’). Both Zfh1-positive CySCs and Eya-positive cells were observed (see Fig. 7C’-C’’, Table 3) in 100% (16/16) cases and no abnormal GSC differentiation was observed. These results suggest eIF4B plays no role in the cyst cells, however due to the possible inefficiency of the knockdown and one cannot conclude that the protein was indeed absent. eIF4H isoforms are involved in the assembly of the eIF4F complex and activate eIF4A (Wu *et al.*, 2011; Vaysse *et al.*, 2015). However, knocking down two *Drosophila eIF4H* paralogues, *eIF4H1* (see Fig. 7E-E’’’) and *eIF4H2* (see Fig. 7F-F’’’) in cyst cells resulted in a control phenotype (see Fig. 7A-A’’’, Table 3). I observed Zfh1-positive and Eya-positive cells in 100% of testes (see Fig. 7E’-E’’, 7F-F”), no ectopic CySCs (see Fig. 7E’, Fig. 7F’) and normal Topro staining (see Fig. 7E’’’, Fig. 7F’’’). These results suggest eIF4H1/2 are not required in CySC self-renewal or differentiation. eIF4G is a crucial scaffold protein involved in the assembly of eIF4F and interacts with the 43S PIC complex (Sonenberg and Hinnebusch 2009). Knocking down eIF4G in the cyst lineage (see Fig. 7D, Table 3) led to a complete loss of Zfh1-positive CySCs in 77% (15/20) of testes (see Fig. 7D’), and loss of the hub in 94% (19/20) of testes (see Fig. 7D’’). Only Eya-positive cells remained (see

Fig. 7D'') and abnormal GSC differentiation was also observed in 100% of testes (see Fig. 7D''').

In summary, eIF4E1, eIF4A and eIF4G share the same phenotype, characterised by a complete depletion of Zfh1-positive CySCs (see Tables 2 & 3), which implies a role in the self-renewal of CySCs. Due to control-like phenotypes resulting from knocking down eIF4B, eIF4H1 and eIF4H2 one is unable to conclude any specific roles for these factors in CySC fate. However, one cannot distinguish between a true lack of function and an inefficient knockdown without further experiments. Overall these results suggest a role for the eIF4F complex in maintaining CySCs.

Figure 7: eIF4A and eIF4G knockdowns lead to loss of CySCs

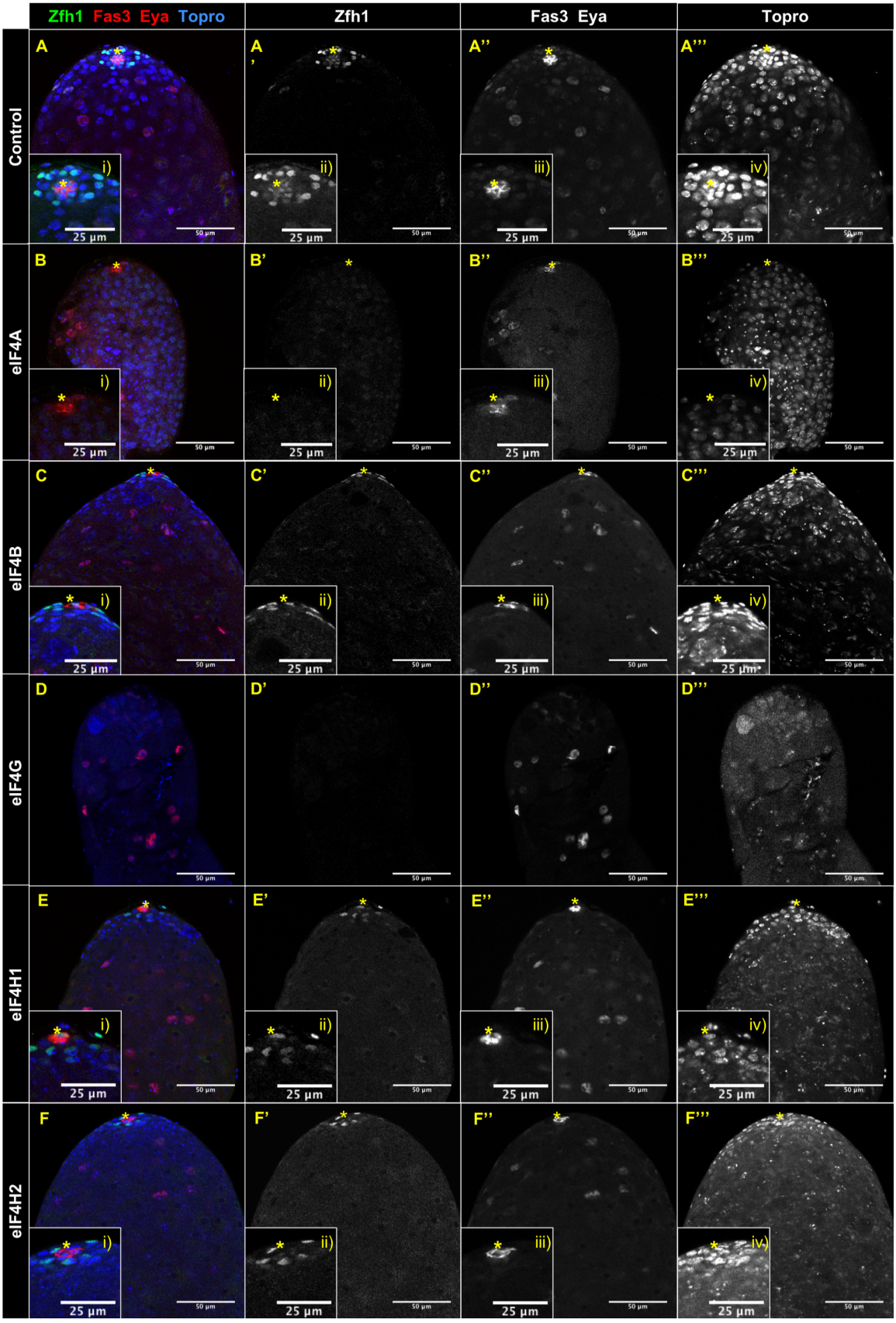


Figure 7 Legend: eIF4A and eIF4G knockdowns leads to loss of CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). Images A through to D''' have 50um scale bars displaying the testis apex and images i)-iv) of each genotype display a focus on the niche (asterisk) with a scale bar of 25um. Control testes (A-A''') had all cell types present (A, green), including Zfh1-expressing CySCs (A, i), A', ii), green), Eya-expressing cyst cells (A'', red) and a Fas-3 expressing hub (A'', asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A''', blue). Knocking down eIF4A (B-B''') led to a complete depletion of Zfh-1 expressing CySCs (B, B', ii), green), with only Eya-expressing cyst cells remaining (B, B'', red) and impaired GSC differentiation, due to an unusual Topro stain (B''', blue). This suggests a role in CySC self-renewal. Knocking down eIF4B (C-C'''), eIF4H1 (E-E''') and eIF4H2 (F-F''') led to control-like phenotypes, with all cell populations in tact; including Zfh1-expressing cells (green), Eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue), evoking no role for these factors in either CySC self-renewal or differentiation. Knocking down eIF4G (D-D''') led to a complete depletion of Zfh1-expressing CySCs (D, D', green), with only Eya-expressing cyst cells remaining (D, D'', red) and impaired GSC differentiation, due to unusual Topro staining (D, D''', blue). This suggests a role for eIF4G in the self-renewal of CySCs.

3.2.3 eIF3 promotes cyst cell differentiation

Next, I examined testes in which eIF3 subunits were knocked down. eIF3 interacts with eIF4G and with the small subunit of the ribosome to bring the PIC to the 5' end of the mRNA (Sonenberg and Hinnebusch 2009). Since eIF3 interacts with eIF4F, it is likely that they play similar roles in CySC fate, and eIF3 component knockdowns could result in loss of Zfh1-expressing CySCs similar to eIF4F knockdowns. I assessed knockdowns of eIF3 subunit b (eIF3b/eIF3-S9) and eIF3 subunit a (eIF3a/eIF3-S10). Surprisingly, in testes in which eIF3b was knocked down (see Fig. 8B-B'", Table 3) I observed ectopic Zfh1-positive cells outside the niche in 82% (25/31) of testes (see Fig. 8B') and increased Topro staining in 93% (28/31) of testes (see Fig. 8B'"), implying that GSCs were unable to differentiate. Similarly, eIF3a knockdown (see Fig. 8C, Table 3) led to a gain of Zfh1-positive cells, and Zfh1-expressing cells being found outside the niche in 100% (15/15) of testes (see Fig. 8C'). Moreover, Eya-positive cells were absent in 7% (1/15) of testes (see Table 3) and increased Topro staining in 100% of testes (see Fig. 8C'"), implying that GSCs were also unable to differentiate. Additionally, in 20% (3/15) of testes, knocking down of eIF3a led to increased hub size (see Fig. 8C'').

Both knockdowns shared similar phenotypes with ectopic Zfh1-positive cells away from the niche (see Tables 2 & 3), which suggested a role for the eIF3 complex in promoting differentiation, a surprising result considering the results of knocking down eIF4F components. Based on my findings, eIF3 may play a role in the differentiation of cyst cells and may be able act independently of eIF4F (see Fig. 6 & Fig. 7) in the cyst lineage.

Figure 8: eIF3 knockdown leads to ectopic CySCs

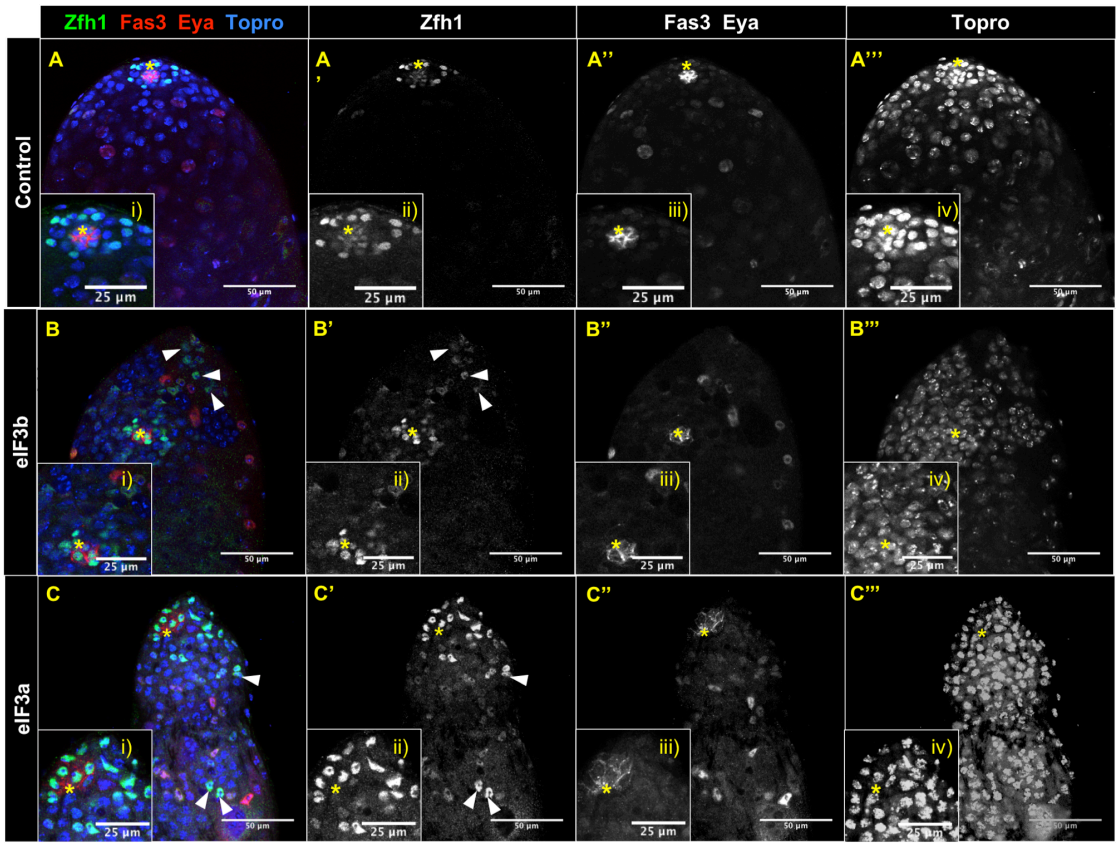


Figure 8 Legend: eIF3 knockdown leads to ectopic CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). Images A through to C''' have 50um scale bars displaying the testis apex and images i)-iv) of each genotype display a focus on the niche (asterisk) with a scale bar of 25um. Control testes (A-A''') had all cell types present (A, green), including Zfh1-expressing CySCs (A, i), A', ii), green), Eya-expressing cyst cells (A'', red) and a Fas-3 expressing hub (A'', asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A''', blue). Knocking down eIF3b (B-B'''), led to Zfh1-expressing CySCs (B, B', green) to increase in number and were found outside the niche (asterisk, red) (B, B', green, arrows). In addition, there was impaired GSC differentiation, due to excess Topro staining (B, B''', blue), all of which conveys a role in cyst cell differentiation. A similar phenotype was observed in the eIF3a knockdown (C-C'''), with increased numbers of Zfh1-expressing CySCs (C, C', green), which were also found outside the hub (asterisk, red) (C, C'', green, arrows). Topro staining also implied that there was impaired GSC differentiation (C, C''', blue). eIF3a may thus also play a role in cyst cell differentiation.

3.2.4 eIF1A promotes cyst cell differentiation

Next, I examined the phenotypes of eIF1 and eIF1A knockdowns. eIF1 and eIF1A cooperatively bind to the light subunit of the ribosome (40S), which associates with eIF3, altogether forming the 43S PIC (Sonenberg and Hinnebusch 2009). Since, knocking down eIF3 components lead to gain of Zfh1-expression phenotypes, I expected the knockdown of eIF1 and eIF1A to have a similar effect. In testes in which eIF1 was knocked down using *Tj-Gal4* (see Fig. 9B-B'''), resulted in a control phenotype in 100% (18/18) of testes (see Table 3). eIF1A knockdown (see Fig. 9C-C''', Table 3) led to a different phenotype, with Zfh1-positive cells present outside the niche in 100% (16/16) of testes (see Fig. 9C') and an additional loss of Eya-positive cells in 32% (5/16) of testes (see Table 3). I also recorded increased Topro staining in 100% of testes (see Table 3), implying an impaired ability for GSCs to differentiate (see Fig. 9C''').

Knocking down eIF1A led to the presence of ectopic Zfh-1expressing cells (see Table 2), similar to eIF3 knockdowns, and contrary to eIF4F knockdowns where Zfh1-expressing cells were lost (see Fig. 6, 7, 8). Knocking down eIF1 led to control-like phenotypes, with no obvious defects. These results suggest that eIF1A may play a role in the differentiation of cyst cells however one cannot conclude a role for eIF1.

Figure 9: eIF1A knockdown leads to ectopic CySCs

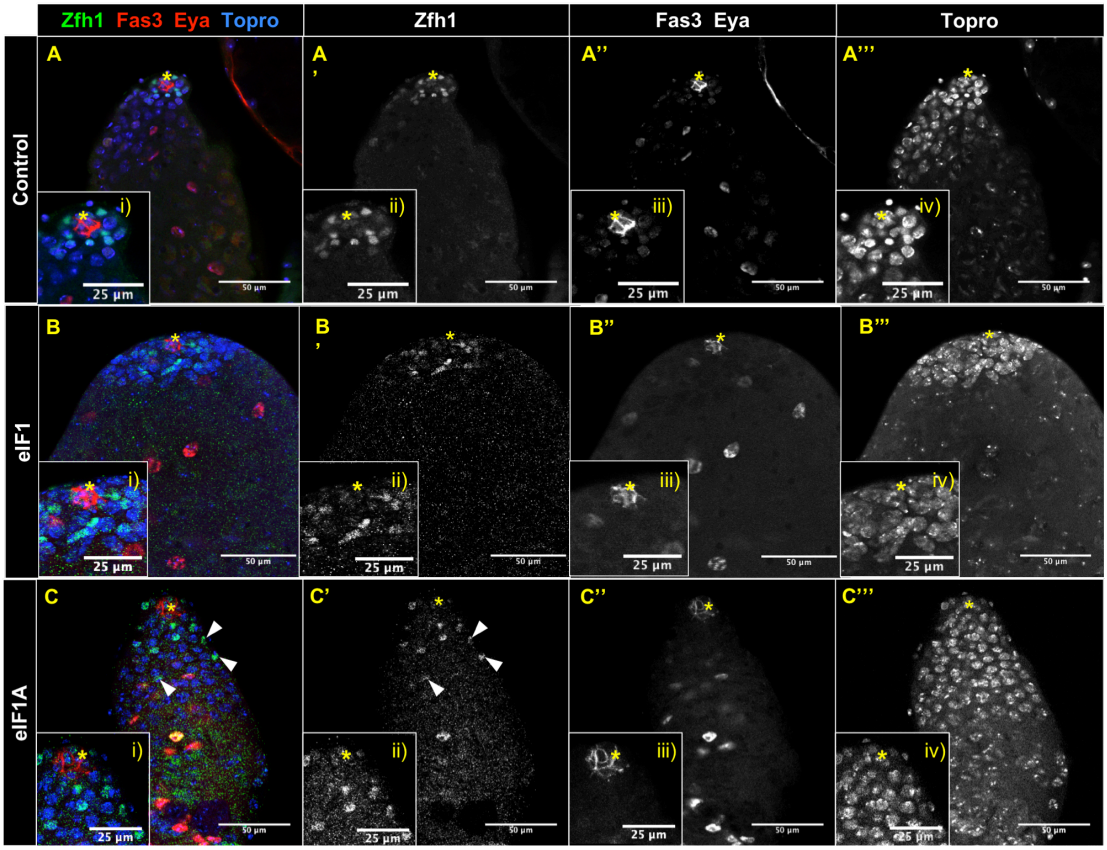


Figure 9 Legend: eIF1 knockdown leads to ectopic CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). Images A through to C''' have 50um scale bars displaying the testis apex and images i)-iv) of each genotype display a focus on the niche (asterisk) with a scale bar of 25um. Control testes (A-A''') had all cell types present (A, green), including Zfh1-expressing CySCs (A, i), A', ii), green), Eya-expressing cyst cells (A'', red) and a Fas-3 expressing hub (A'', asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A''', blue). Knocking down eIF1 led to a control-like phenotype (B-B'''), with all cell populations in tact; including Zfh1-expressing cells (green), Eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue), evoking no role for these factors in either CySC self-renewal or differentiation. Knocking down eIF1A (C-C''') led to Zfh1-expressing CySCs to increase in umber and be found outside the niche (asterisk, red) (C, C'', green, arrows). Topro staining also implied that there was impaired GSC differentiation (C, C''', blue). This implies a role for eIF1A in cyst cell differentiation.

3.2.5 eIF2 and eIF2B promote cyst cell differentiation

eIF2alpha and eIF2gamma function as part of a heterotrimer (eIF2) with eIF2beta. eIF2alpha and eIF2gamma mediate the binding of tRNA to the 40S subunit, in a guanosine triphosphate (GTP)-dependent manner (Sonenberg and Hinnebusch, 2009a; Voigts-Hoffmann, Klinge and Ban, 2012). eIF2Balpha is a subunit of a five-subunit complex eIF2B, which catalyses guanine nucleotide exchange on eIF2, to regulate its activity (Yang and Hinnebusch, 1996; Dev *et al.*, 2009). Based on this I hypothesised that knocking down these factors would lead to similar phenotypes to those caused by knocking down eIF3 and eIF1. Knocking down eIF2alpha (see Fig. 10B, Table 3) led to CySCs being observed outside of the niche in 86% (14/16) of testes (see Fig. 10B'). In addition, GSC differentiation was impaired in 94% (15/16) of testes (see Fig. 10B'''). eIF2gamma knockdown (see Fig. 10C-C''', Table 3) led to ectopic Zfh1-expressing CySCs outside of the niche in 60% (9/15) of testes (see Fig. 10C') and abnormal GSC differentiation in 100% of testes (see Fig. 10C'''). The eIF2Balpha knockdown (see Fig. 10D-D''', Table 3) also showed an increase in CySCs with Zfh1-positive cells present outside the niche in 11% (2/18) of testes (see Fig. 10D') and a general increase in CySC number compared to the control (see Fig. 10D'ii), Fig. 10A'ii)).

All knockdowns were classed as gain of CySC phenotypes (Tables 2 & 3). Based on these phenotypes, eIF2 alpha, eIF2gamma and eIF2Balpha may play a role in the differentiating cyst cells.

Figure 10: eIF2 and eIF2B knockdown leads to ectopic CySCs

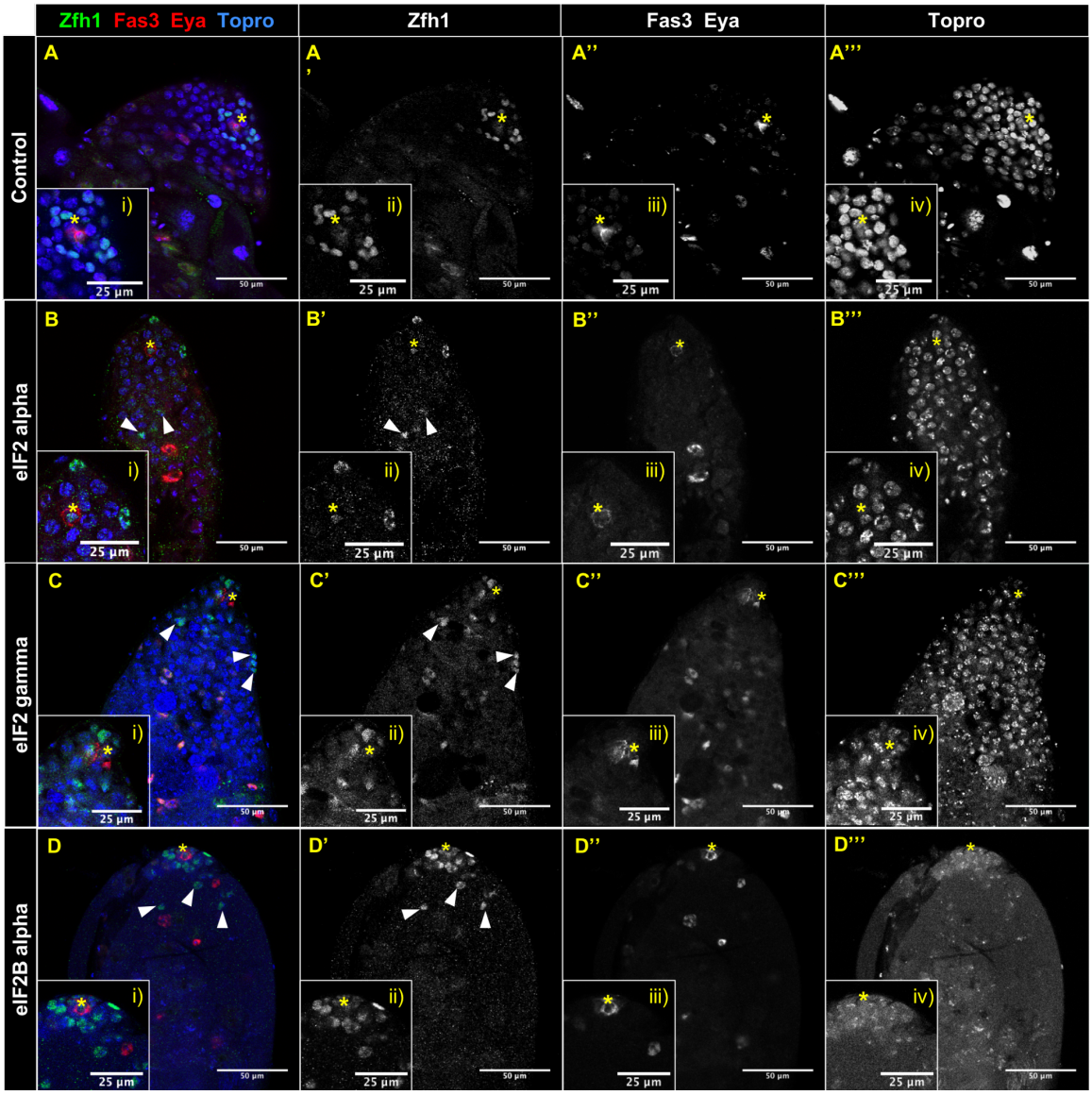


Figure 10 Legend: eIF2 and eIF2B knockdown lead to ectopic CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). Images A through to D''' have 50um scale bars displaying the testis apex and images i)-iv) of each genotype display a focus on the niche (asterisk) with a scale bar of 25um. Control testes (A-A''') had all cell types present (A, green), including Zfh1-expressing CySCs (A, i), A', ii), green), Eya-expressing cyst cells (A'', red) and a Fas-3 expressing hub (A'', asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A''', blue). Knocking down eIF2alpha (B-B''') led to Zfh1-expressing CySCs to increase in number (B, B', green) as well as be found outside the niche (asterisk, red) (B, B', green, arrows). Topro staining also implied that there was impaired GSC differentiation (B, B''', blue). eIF2alpha may therefore play a role in cyst cell differentiation. A similar phenotype is observed in the eIF2gamma knockdown (C-C'''), with Zfh1-expressing CySCs increasing in numbers (C, C', green) and being found outside the niche (asterisk, red) (C, C', green, arrows). Impaired GSC differentiation is evoked through excessive Topro staining (C, C''', blue). This also indicates a role for eIF2gamma in differentiation. Knocking down eIF2Balpha (D-D''') led to Zfh1-expressing cells increasing in number to the increased numbers (D, D', green) and being outside the niche (D, D', green, arrows), along with impaired GSC differentiation (D, D''', blue) in the knockdown phenotype. eIF2Balpha may thus also play a role in cyst cell differentiation.

Overall, the first part of the screen identified a potential role for translation in fate of CySCs. I conclude that all subunits of eIF4F are required for CySC self-renewal, with the exception of eIF4E6, while eIF3/2/1 promote cyst cell differentiation. The fact that eIF4F components act differently from other initiation factors in CySC fate maintenance is suprising, and implies that translation is regulated differently in self-renewal and differentiation. Self-renewal requires the presence of eIF4F, since knockdown of eIF4F subunits results in loss of Zfh1-expressing CySCs, while conversely, differentiation uses eIF3, eIF1 and eIF2, due to loss of any of these results in ectopic Zfh1-expressing cell away from the hub. Since eIF4F binds the 5' cap and assembles the initiation complex at the 5' end of the mRNA, one plausible explanation is that 5' cap-mediated translation is required in CySCs but not during differentiation. This suggests that a switch in the mode of translation might occur during CySC differentiation. These results therefore lead to two important questions: i) what causes the requirement for eIF4F to change in the translation from CySCs to differentiated cells and ii) how is translation initiation controlled in differentiating cyst cells if it does not involve eIF4F binding to the 5' cap.

3.3 RNAi Knockdown Screen: Non-canonical Translation Initiation Factors

Although the majority of mRNA is translated via the canonical cap-dependent mechanism, eukaryotic cells have evolved at least 5 alternative initiation mechanisms. One of these mechanisms involves specific mRNA structures known as Internal ribosomal entry sites (IRES), which recruit the ribosome independently of most cap-dependent factors. IRES initiation can use IRES trans-activating factors (ITAFs), which help scaffold other factors and recruit the ribosomal subunits (Sonenberg and Hinnebusch 2007; Mitchell and Parker 2015; Komar and Hatzoglou 2011). Additional mechanisms include m^6A modifications, which stimulate translation in cases of uncapped mRNAs (Meyer *et al.*, 2015; Mitchell and Parker, 2015). 5'UTR M^6A modification of mRNA and YTHDF1-bound mRNAs promote the recruitment of ribosomal subunits and initiate translation (Meyer *et al.*, 2015; Mitchell and Parker, 2015; Shi *et al.*, 2017). Here, I generated knockdowns of proteins that I selected based on their known involvement in alternative mechanisms of translation initiation: pAbp, GlyRS, eIF6, CG7482, eIF2D, DENR, sqd, heph, unr, syp, Hrb87F, La, Larp, YTHDF, YTHDC1 and SAM-S (Lasko, 2000; Komar and Hatzoglou, 2011b; Lu *et al.*, 2015; Mitchell and Parker, 2015; Marygold, Attrill and Lasko, 2017). These factors

have mRNA binding ability and perform functions in non-canonical translation initiation methods, such as IRES translation initiation (see Fig. 2) (Lasko, 2000).

I knocked down these factors using the same Gal4-UAS system to express a UAS-RNAi transgene for each factor specifically in the cyst cell lineage and followed the same breeding methods as previously mentioned for cap-dependent factor knockdowns. The flies were dissected and the testes immunostained using Zfh1 (CySCs), Fasciclin III (Fas3) (hub), Eya (differentiated cyst cells) and Topro-3-iodide (Topro) (a DNA stain to label all cell nuclei). I scored phenotypes in the exact way as previously shown, scoring for CySCs, ectopic CySCs, cyst cells, the hub and GSC differentiation, however the latter was not always possible due to inconsistent stain quality. The same control, carrying *Tj-Gal4* and *Gal80*^{ts}, (*yw122;Tj-Gal4/+;Tub,Gal80*^{Ts}*/+)* is used. A summary of these results can be found in Tables 2 and 3.

Other than identifying a role for translation in CySC fate, the contrasting phenotypes of eIF4F complex knockdowns compared to other complexes associated with 5' cap-dependent initiation of translation, i.e. eIF1, eIF2 and eIF3, demonstrates that different complexes may individually be involved in specifying different cell fates (i.e. stem cell vs differentiating cell). Assessing the phenotypes of non-canonical factor knockdowns would allow me to determine whether stem cell fate could be regulated by alternative translation initiation methods.

3.3.1 pAbp and GlyRS promote cyst cell differentiation

Firstly, I investigated factors, which promote translation initiation in both canonical and non-canonical mechanisms. poly(A) binding protein (pAbp) is a component of the mRNA-protein complex involved in translation initiation. It binds to the polyadenylated 3' end (poly(A)) of an mRNA molecule. This promotes stability and inhibits nonsense-mediated mRNA (Berlanga, Baass and Sonenberg, 2006; Hinnebusch, Ivanov and Sonenberg, 2016). pAbp is also known to physically interact with eIF4G, which promotes cap-dependent initiation of translation and the circularisation of mRNA (Tarun and Sachs, 1996; Berlanga, Baass and Sonenberg, 2006). pAbp also binds other factors such as eIF3 and ITAFs, suggesting it is not restricted to canonical, cap-dependent initiation of translation (Komar and Hatzoglou 2011). Knocking down pAbp (see Fig. 11B-B''', Table 3) led to Zfh1-positive CySCs

being observed outside the niche in 96% (24/25) of testes (see Fig. 11B'). All other cells were present in 100% of testes (see Fig. 11B'', 11B'''), but due to ectopic CySCs, I classed the phenotype as a gain of Zfh1-positive cells (see Table 2). pAbp may thus play a role in differentiation.

Glycyl-tRNA synthetase (GlyRS) belongs to a family of aminoacyl-tRNA synthetases which catalyse the ligation of amino acids to their cognate tRNAs (Lu *et al.*, 2015). GlyRS is important for all mechanisms of translation initiation, catalysing an aminoacylation reaction, which combines a tRNA molecule with a respective amino acid. Knockdown of GlyRS resulted in (see Fig. 11C-C''', Table 2) CySCs (see Fig. 11C'), cyst cells and the hub all being present (see Fig. 11C''), as well as ectopic CySCs in 40% (6/15) of testes (see Fig. 11C-C'). I therefore classed this phenotype as a gain of Zfh1-positive phenotype (see Table 2). GlyRS knockdown suggests a role in cyst cell differentiation.

Eukaryotic translation initiation factor 6 (eIF6) is a ribosome anti-association factor, which binds to the 60S ribosomal subunit and prevents its associate with the 40S subunit, thus impeding a premature formation of the 80S without mRNA (Brina *et al.*, 2015). It is also a critical factor in rRNA processing and ribosomal biogenesis, making it a widespread regulator of translation (Benelli *et al.*, 2012). Upon knocking down eIF6 (see Fig. 11D-E''', Table 3), I observed the loss of CySCs in 42% (7/15) of testes (see Fig. 11E') and the hub in 48% of testes (7/15) (see Fig. 11E''). Additionally, I observed ectopic CySCs in 35% (5/15) (see Fig. 11D') of testes. Due to these phenotypes I classed the knockdown phenotypes as unclear (see Table 2).

CG7483, also known as eIF4III, encodes a helicase (Barbosa *et al.*, 2012), which aids in localising RNA and promoting the decay of nonsense-mediated mRNA, as well as in pre-mRNA splicing (Palacios *et al.*, 2004). Knocking down this factor led to ectopic CySCs being observed in 70% (12/17) of testes (see Fig. 11F'). Therefore, CG7483 is required for differentiation (see Fig. 11F-F''', Table 3).

Finally, I knocked down Eukaryotic initiation factor 2D (eIF2D), also known as ligatin, which is involved in cytoplasmic translation recycling and re-initiation. It functions in disassembling the ribosome-mRNA-tRNA complex following termination and then reinitiating the process (Jackson, Hellen and Pestova, 2012). eIF2D also associates with DENR-MCT-1 to promote re-initiation through ribosome disassembly (Marygold, Attrill and Lasko, 2017). eIF2D has been associated with cap-independent translation

initiation methods in the place of eIF2 (Marygold, Attrill and Lasko, 2017). Knock down of eIF2D resulted in a normal complement of Zfh1-expressing cells being present at the niche, as well as Eya-expressing differentiated cells in 100% of testes (see Fig. 11A-A'', Table 3). This suggests that either the RNAi was not efficient or eIF2D is not required for fate specification in the cyst cell lineage.

In conclusion knocking down pAbp, GlyRS and CG7482 leads to gain of CySC phenotypes (see Tables 2 & 3) suggesting that these factors are all required for normal cyst cell differentiation. Knocking down eIF6 caused an unclear phenotype, leading to no clear conclusions and knocking down eIF2D resulted in a control phenotype, suggesting either incomplete knockdown or no requirement for eIF2D in CySC fate (see Tables 2 & 3).

Figure 11: Knocking down pAbp and GlyRS leads to ectopic CySCs

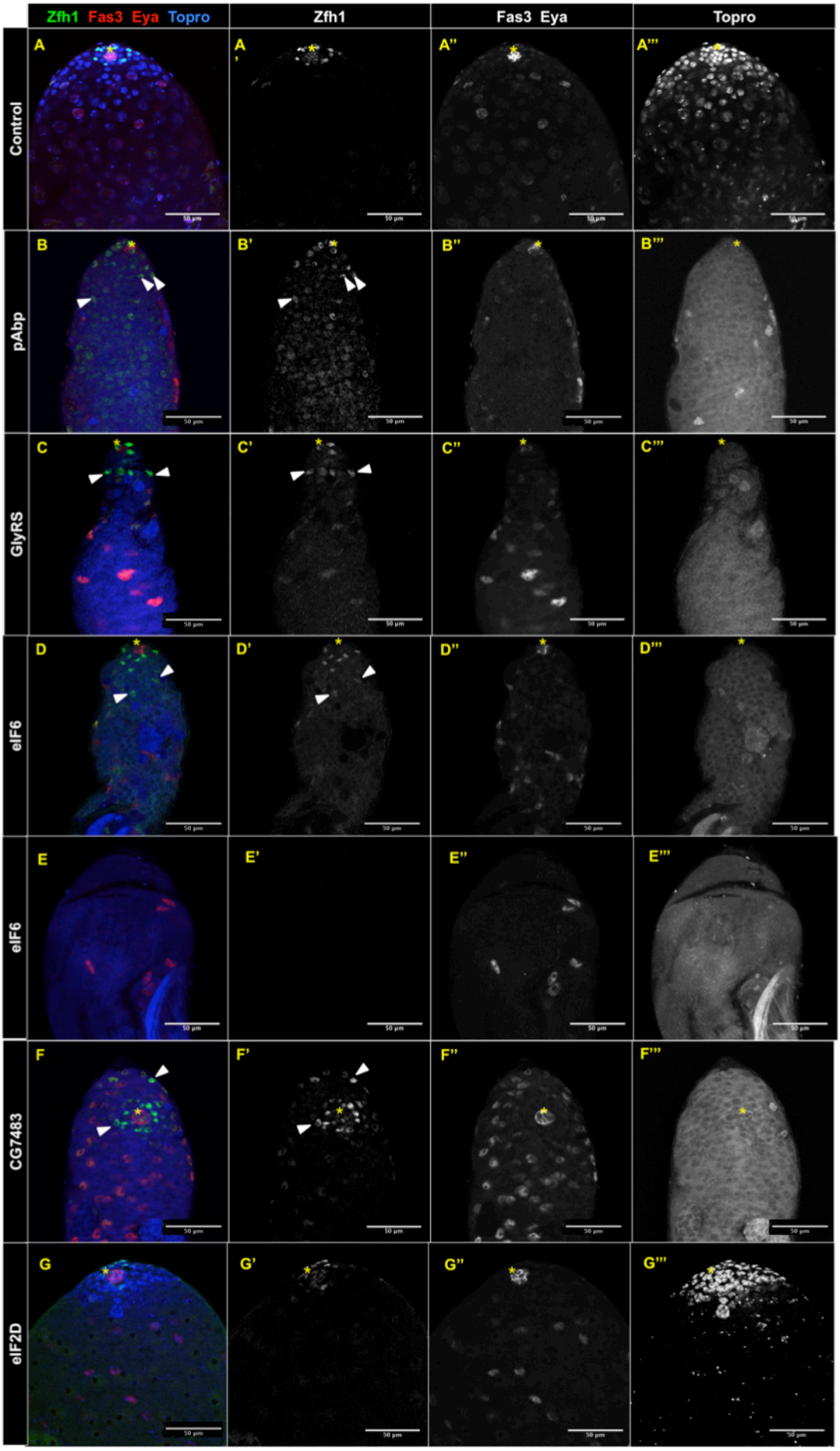


Figure 10 Legend: Knocking down pAbp and GlyRS leads to ectopic CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). All images have 50um scale bars displaying the testis apex. Control testes (A-A'") had all cell types present (A, green), including Zfh1-expressing CySCs (A, A', green), Eya-expressing cyst cells (A", red) and a Fas-3 expressing hub (A", asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A'", blue). Knocking down pAbp (B-B'") led to Zfh1-expressing CySCs increasing in number (B, B', green) and being found outside the niche (asterisk, red) (B, B', green, arrows). GSC differentiation could not be assessed due to unsuccessful Topro stains (B'", blue). However, pAbp still demonstrated a role in cyst cell differentiation. Knocking down GlyRS (C-C'") led to Zfh1-expressing CySCs increasing in number with (C, C', green) and being found outside the hub (asterisk, red) (C, C", green, arrows), indicating a role in differentiation. Knocking down eIF6 (D-E'") led to Zfh1-expressing CySCs increasing in number (D, D', green) in some testes and a complete depletion of Zfh1-expressing CySCs (E, E', green) in others, causing the role of eIF6 to be unclear. Knocking down CG7483 (F-F'") led to Zfh1-expressing CySCs increasing in number (F', green) and being found outside the niche (F, F', green, arrows), evoking a role in cyst cell differentiation. Knocking down eIF2D (G-G'") led to a control-like phenotype, with all cell populations in tact; including Zfh1-expressing cells (green), Eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue), evoking no role for these factors in either CySC self-renewal or differentiation.

3.3.2 Knock down of IRES trans-activating factors and related factors shows little effect on CySC fate other than Hrb87F which promotes cyst cell differentiation

Switching between alternative initiation mechanisms can regulate translation initiation and gene expression. Some mRNAs contain specific Internal ribosomal entry sites (IRES) structures, which can recruit ribosomal subunits. Recruitment can occur independently or with the help of initiation factors, such as eIF3 or ITAFs that are recruited to the site (Mitchell and Parker, 2015). Here, I investigated whether IRES-trans-activating factors (ITAFs) and IRES-associated factors play a role in regulating CySC fate. By extension, this would also allow me to see whether different translation initiation mechanisms regulated gene expression programs in the cyst lineage. Since eIF4F complex is not required during differentiation, but eIF3 is, translation initiation during differentiation could be cap-independent, and might depend on IRES translation. Therefore, knocking down factors that promote IRES initiation could mimic eIF3 knockdown and lead to ectopic self-renewing cells.

Density regulated protein (DENR) is a non-canonical translation initiation factor, promoting re-initiation of ribosomes through association with multiple copies in T-cell lymphoma-1 (MCT-1) (Marygold, Attrill, and Lasko 2017; Komar and Hatzoglou 2011). Further research in flies has identified a role for DENR in translation re-initiation and selectively promotes the translation of mRNAs containing upstream open reading frames (uORFs) (Marygold, Attrill and Lasko, 2017). In the context of IRES, DENR-MCT-1 along with Ligatin or eIF2D also help in delivering methylated tRNA (Met-tRNA^{Met}) to the 40S ribosomal subunit in the absence of eIF2 (Komar and Hatzoglou 2011). I observed CySCs and cyst cells (see Fig. 12A-A’’’) in 100% (18/18) of testes (see Fig. 12B-B’’’, Table 3) and ectopic Zfh1-expressing cells in 1 testis (see Fig. 12C’). I decided to class the phenotype as unclear (see Table 3) due to the fact only one testis displayed a different phenotype. Based on these results, I was unable to conclude a role for DENR in CySC fate. However, it would be worth repeating this experiment with an independent RNAi line against DENR to determine whether it is playing a role in cyst cell differentiation.

Squid (sqd) is an heterogeneous nuclear ribonucleoprotein hnRNP), a family of RNA binding proteins that perform a role in the localisation of mRNA as well as a regulatory one in translation (Geuens, Bouhy and Timmerman, 2016). hnRNP proteins have been characterised as ITAFs in (Thakor *et al.*, 2017), and promote IRES-dependent translation initiation. Sqd has multiple functions in both cap-

dependent and cap-independent translation initiation mechanisms. Amongst many physical interactions, sqd has been shown to interact with multiple translation regulators including: Unr (Mihailovic *et al.*, 2012), pAbp (Clouse, Ferguson and Schüpbach, 2008), Hrb87F (Matunis, Matunis and Dreyfuss, 1992) and syp (McDermott *et al.*, 2012), making it an interesting candidate for the screen. Testes in which sqd was knocked down in the cyst lineage (see Fig. 12D-D'''', Table 3), showed no defects compared to the control (see Fig. 12A-A'''). Zfh1-expressing CySCs (see Fig. 12D'), Eya-positive cyst cells (see Fig. 12D'') and Fas3-expressing hubs (see Fig. 12D'') were present in 100% (16/16) of testes (see Table 3). Thus, I could not draw any conclusions as to whether sqd plays a role in CySC self-renewal or differentiation.

Syncrip (syp) is an hnRNP that generally regulates localized translation in the synapse of neuromuscular junctions (McDermott *et al.*, 2012, 2014). The protein binds RNA and is known to interact with sqd (McDermott *et al.*, 2012) and Pten (a regulator of the PI3K/Tor pathway) (Vinayagam *et al.*, 2016), making it an interesting candidate for the screen. Knocking syp (see Fig. 12G-G'''), led to Zfh1-expressing CySCs being found outside the niche in 26.3% (6/21) of testes (see Fig. 12G-G') and yet also a complete depletion of hub cells in 9.6% (2/21) of testes. This led to the factor to be classed as unclear (see Table 2).

Hephaestus (*heph*) encodes a polypyrimidine tract-binding protein (PTB), which also belongs to the hnRNP family (Besse *et al.*, 2009). Amongst various functions this RNA-binding protein plays a role in regulating mRNA localisation and splicing as well as in the regulation of IRES translation initiation (Lasko, 2000; Besse *et al.*, 2009). Knocking down this factor produced a control phenotype in 100% (24/24) of testes (see Fig. 12E-E''', Table 3). This implied no conclusive role for the factor in CySC fate.

Upstream of N-ras (Unr) is an RNA binding protein that performs a chaperoning role for mRNA and regulates translation initiation through IRES. It has been characterised as an ITAF with Apaf-1 as an IRES target (Mitchell *et al.*, 2003). Amongst many interactions, Unr is known to interact with pAbp (Duncan, Strein and Hentze, 2009), and sqd (Mihailovic *et al.*, 2012), causing it to be a versatile regulator of translation. When knocking down Unr I did not observe any significant differences from the control samples, and Zfh1-, Eya- and Fas3-expressing cells (see Fig. 12F-F''') were present in 100% (17/17) cases and in their normal expression domains (see Table

3). Based on these results I was also unable to conclude a role for this protein in CySC self-renewal or differentiation.

Heterogeneous nuclear ribonucleoprotein at 87F (Hrb87F) has been characterised by (Lasko, 2000), to be a significant RNA binding protein involved in translation regulation. It interacts physically with sqd (Matunis, Matunis and Dreyfuss, 1992), displaying a role in non-canonical translation initiation. Hrb87F is the homolog of hnRNP A1, an ITAF that regulates the translation of c-myc and cyclin D1 (Gao, Dhar and Bedford, 2017). Knocking down Hrb87F (see Fig. 12H-H'", Table 3) led to ectopic Zfh1-expressing cells being observed in 94% (15/16) of testes (see Fig. 12H') and is therefore required in cyst cell differentiation. Since Hrb87F is ITAF, responsible in the regulation of IRES-mediated translation and promotes CySC differentiation, and eIF4F regulates cap-dependent translation initiation and CySC self-renewal, this could explain how cyst cell differentiation relies on alternative translation initiation methods to maintain that fate.

La autoantigen protein (La) binds the 3'hydroxyl termini of specific mRNAs (Yoo and Wolin, 1994) and has been characterised as an ITAF which aids the process of IRES as an alternative translation initiation (Yoo and Wolin, 1994; Lasko, 2000; Blagden *et al.*, 2009). Upon knockdown I observed a control phenotype (see Fig. 12I-I'", Table 3), with an intact hub, CySCs and cyst cells present in 100% (17/17) of testes (see Table 3). Based on these findings, I could not conclude a role for La in CySC self-renewal or differentiation.

La related protein (Larp) has been suggested to participate in translation via regulation of mRNA decay through binding to pAbp (Blagden *et al.*, 2009). Larp has been shown to physically bind and interact with pAbp promoting mRNA stability and by extension promoting translation (Blagden *et al.*, 2009). It additionally interacts with La proteins, ITAFs, which regulate IRES (Yoo and Wolin, 1994). Knocking down Larp1 produced a control phenotype (see Fig. 12J-J'", Table 3), displaying no differences or defects compared to control samples (see Fig. 12A'-A'"). Along with La, I cannot conclude a function for Larp1 in the context of CySC fate.

In summary, IRES-related factor DENR displays an unclear role in CySC fate (see Tables 2 & 3), due to the low penetrance of the gain of Zfh1-expressing cell

phenotype observed in 1/18 testes (see Fig. 12C, Table 3). Knocking down syp also led to an unclear phenotype. Knocking down Hrb87F produced a strong gain of CySC phenotype, suggesting a role in cyst cell differentiation. La, Larp, sqd, heph and Unr produced control phenotypes when knocked down, suggesting no conclusive roles in CySC fate or a lack of efficient knockdown by the RNAi lines used (see Table 2 & 3). One cannot distinguish between a true lack of function and an inefficient knockdown without further experiments. In conclusion, at least one factor, Hrb87F, known to be involved in IRES also has a role in promoting CySC differentiation, although further work will be required to determine whether its role in CySC differentiation is due to its promotion of IRES-dependent translation.

Figure 12: Knocking down Hrb87F leads to ectopic CySCs and knocking down other IRES related factors leads to either unclear or control-like phenotypes

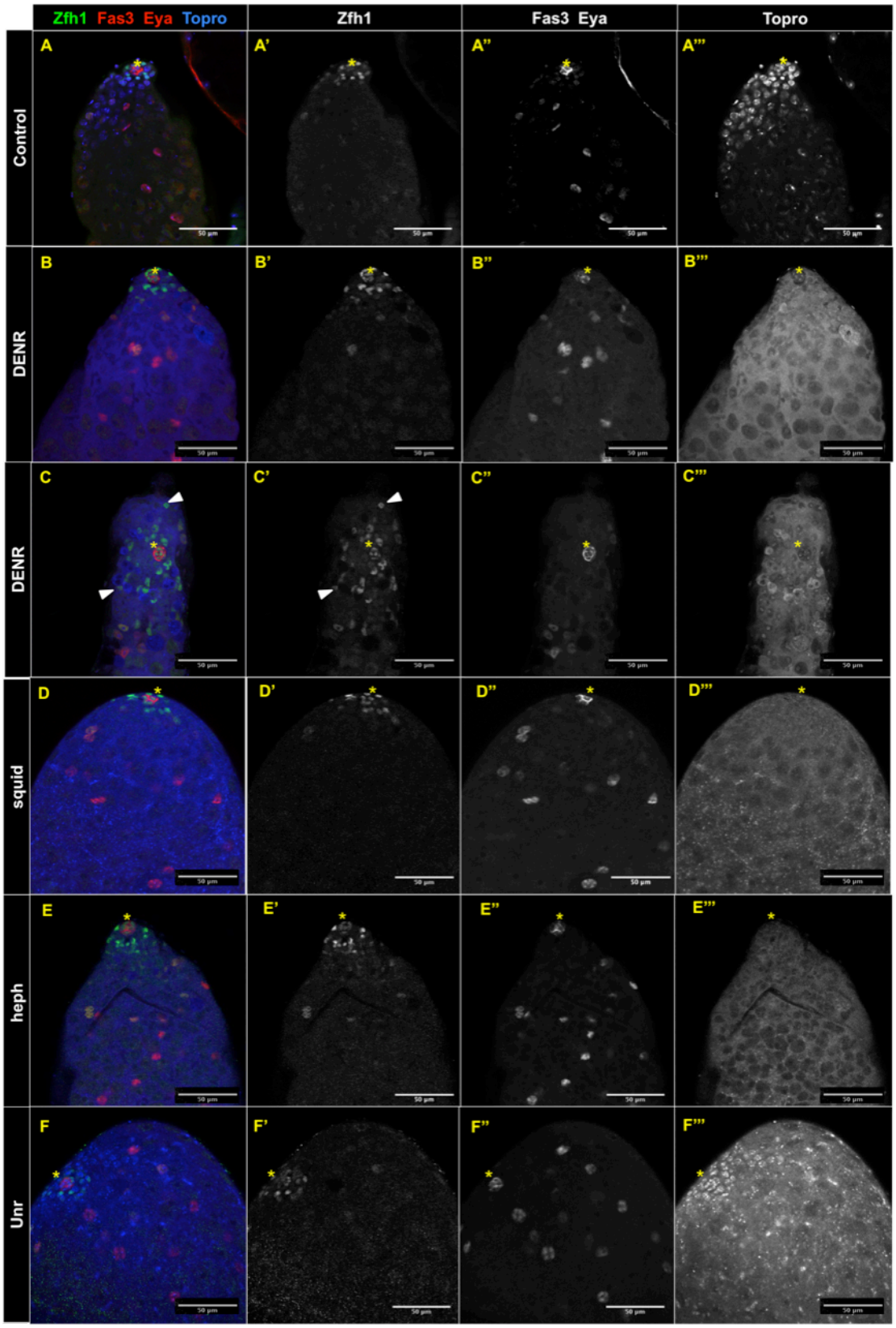


Figure 12: Knocking down Hrb87F leads to ectopic CySCs and knocking down other IRES related factors leads to either unclear or control-like phenotypes

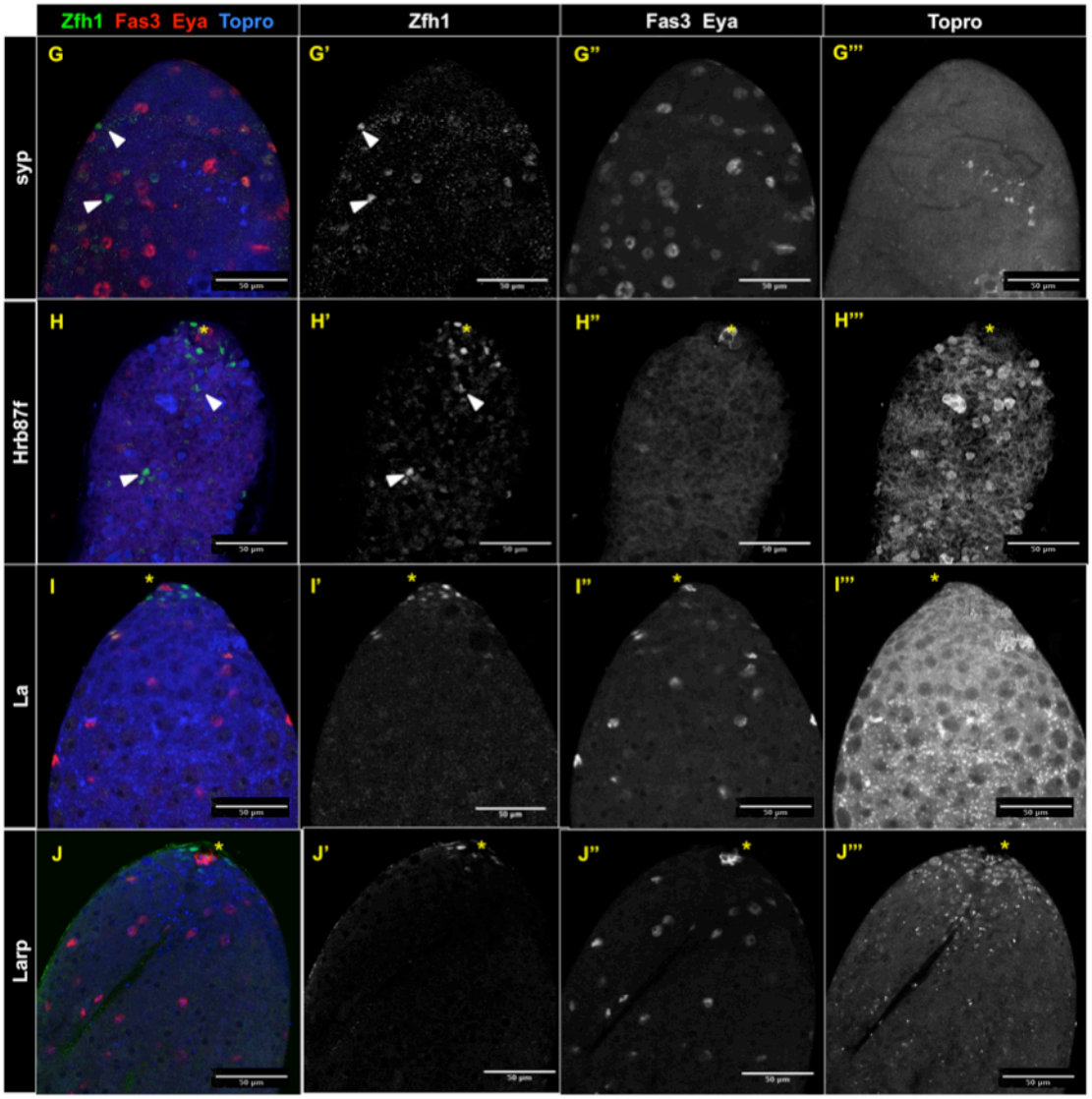


Figure 12 Legend: Knocking down Hrb87F leads to ectopic CySCs, whilst knocking down other IRES related factors leads to either unclear or control-like phenotypes

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). All images have 50um scale bars displaying the testis apex. Control testes (A-A'") had all cell types present (A, green), including Zfh1-expressing CySCs (A, A', green), Eya-expressing cyst cells (A", red) and a Fas-3 expressing hub (A", asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A'", blue). Knocking down DENR (B-B'") led to a control-like (A-A'") with all cell populations in tact; including Zfh1-expressing cells (green), Eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue) in most instances. However, a single DENR knockdown led to Zfh1-expressing cells increasing in number (C, C', green) and being found outside the niche (asterisk, red) (C, C', green, arrows). GSC differentiation could not be assessed due to unsuccessfuly Topro stains (C'", blue). DENR thus shows an unclear role in CySC fate. Knocking down squid (D-D'") and heph (E-E'") and Unr (F-F'") led to a control-like phenotypes, with all cell populations in tact; including Zfh1-expressing cells (green), eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue), evoking no role for these factors in either CySC self-renewal or differentiation. Knocking down syp (G-G'") led to Zfh1-expressing CySCs being found outside the niche (G, G', green, arrows) and the absence of the hub (G, G", no asterisk), causing the phenotype to be classed as unclear. Knocking down Hrb87F (H-H'") led to Zfh1-expressing CySCs increasing in number and being found outside the niche (asterisk, red) (H, H', green, arrows), and impaired GSC differentiation (H, H'", blue), conveying a role in cyst cell differentiation. Knockdown both La (I-I'") and Larp (J-J'") led to control-like phenotypes (A-A'"), with all cell populations in tact; including Zfh1-expressing cells (green), eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue), evoking no role for these factors in either CySC self-renewal or differentiation.

3.3.3 RNA methylation may serve as a regulator of CySC fate

Next, I investigated factors and proteins associated with methylation-regulated translation initiation. 5'UTR m^6A modifications have been shown to stimulate translation in cases of uncapped mRNAs (Meyer *et al.*, 2015; Mitchell and Parker, 2015). m^6A modification of mRNA and YTHDF1-bound mRNAs promotes the recruitment of ribosomal subunits and initiates translation independently of cap-dependent translation factors (Meyer *et al.*, 2015; Mitchell and Parker, 2015). I knocked down YTHDF1, YTHDC1 and SAM-S, to investigate the role of RNA methylation in CySC fate. Due to the possibility that self-renewal is governed by cap-dependent translation initiation and differentiation is governed by cap-independent methods, I predicted that these knockdowns would lead to gain of CySC phenotypes.

YT521-B homology N6-methyladenosine RNA binding protein (YTHDF) contains YTH domains that recognize N6-methyladenosine (m^6A)-modified (methylated) RNA (Kan *et al.*, 2017). The YT521-B homology (YTH) domain is an RNA binding domain that binds to short, single-stranded (ss) RNA sequence motifs (Zhang *et al.*, 2010). m^6A methylation of the 3′ UTR regulates translation through its recruitment and recognition of only select binding proteins including YTHDF and YTHDC proteins. Once bound to m^6A-modified mRNA, YTHDF promotes the recruitment of the ribosome and other factors, subsequently facilitating translation (Mitchell and Parker, 2015; Cui *et al.*, 2017). Testes in which YTHDF1 was knocked down had Zfh1-expressing CySCs and Eya-positive cyst cells in 100% (15/15) of cases (see Fig. 13B-B'', Table 3). Strikingly, I observed ectopic Zfh1-expressing CySCs at a distance from the hub in 73% (11/15) of testes (see Fig. 13B'). Additionally, GSC differentiation was impaired in 100% (15/15) of testes (see Fig. 13B'''). These results suggest that YTHDF1 promotes cyst cell differentiation.

YTH domain containing 1 (YTHDC1), coming from the same family as YTHDF is able to recognise and bind m^6A-modified RNA (along with YTHDF) and contributes to a number of processes including, pre-RNA splicing and cap-independent translation initiation (Cui *et al.*, 2017; Kan *et al.*, 2017). The phenotype resulting from the knockdown of YTHDC1 (see Fig. 13C-C''', Table 3) resembled the control phenotype (see Fig. 13A-A'''). CySCs (see Fig. 13C'), cyst cells (see Fig. 13C'') and the hub (Fig. 13C'') persisted, and GSC differentiation was normal (see Fig. 13C''') in 100% (15/15) of testes.

Finally, I investigated the knockdown effects of S-adenosylmethionine synthetase (SAM-S). SAM-S has been associated with post-translation modification of RNA through methylation (Stojković and Fujimori, 2015). Recent findings have drawn attention to SAM-S in the context of intestinal stem cells. Obata *et al*. identified protein synthesis as a regulator of stem cells whilst screening for putative SAM-dependent methyltransferases (Obata *et al.*, 2018). Knocking down SAM-S led to no specific defects (Fig. 13D-D’’’, Table 3), overall resembling the control phenotype (Fig. 13A-A’’’). I thus scored the phenotype as control-like (see Table 3).

In Summary, knocking down YTHDF1 led to a gain of CySC phenotype (see Fig. 13B-B’’’, Table 3), suggesting that it promotes cyst cell differentiation (see Table 2), and implying a potential regulatory role for RNA methylation. Knocking down YTHDC1 and SAM-S lead to control phenotypes (see Fig. 13C-C’’’, Fig. 13D-D’’’, Table 3), which caused me not to conclude a function nor lack of function for these proteins due to the inefficiency of RNAis (see Table 2).

Figure 13: Knocking down YTHDF leads to ectopic CySCs

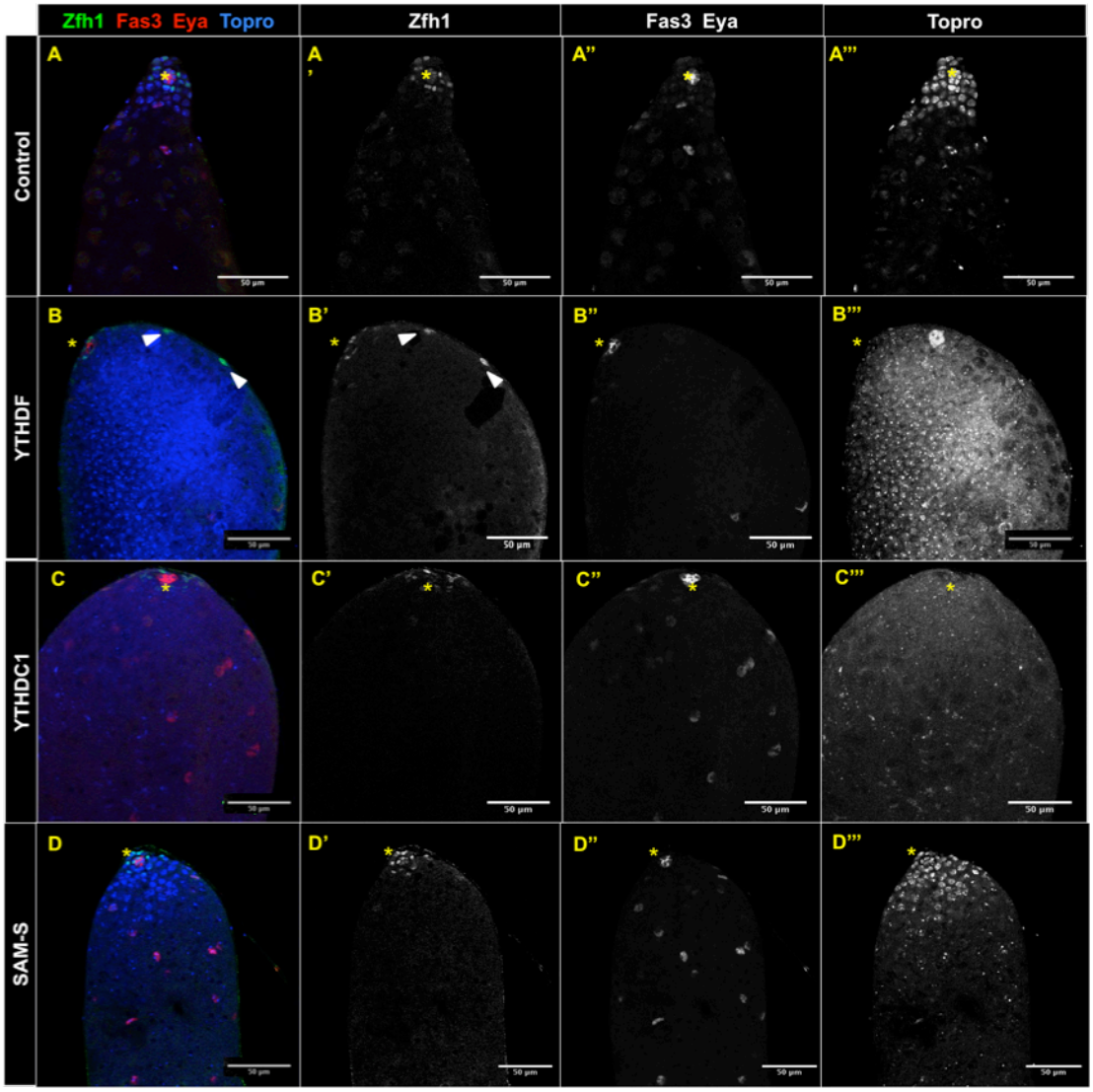


Figure 13 Legend: Knocking down YTHDF leads to ectopic CySCs

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify the hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). All images have 50um scale bars, displaying the testis apex. Control testes (A-A'") had all cell types present (A, green), including Zfh1-expressing CySCs (A, A', green), Eya-expressing cyst cells (A", red) and a Fas-3 expressing hub (A", asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A'", blue). Knocking down YTHDF (B-B'") led to Zfh1-expressing CySCs increasing in number (B-B'") and being found outside the niche (asterisk, red) (B, B', green, arrows), in addition to impaired GSC differentiation (B, B'", blue). YTHDF may thus play a role in CySC differentiation. Knocking down YTHDC1 (C-C'") and SAM-S (D-D'") led to a control-like phenotype, with all cell populations in tact; including Zfh1-expressing cells (green), eya-expressing cyst cells (red) and normal GSC differentiation (Topro, blue), evoking no role for these factors in either CySC self-renewal or differentiation.

Table 2: Translation initiation factor complex categorisation according to knockdown phenotype

	Initiation Mechanism			
Phenotype	**Cap-dependent**	**Non-specific**	**IRES**	**Methylated RNA**
Loss of Zfh1+ CySCs	*4G, 4A, 4E1*			
Gain of Zfh1+ CySCs	*1,1A, 2alpha, 2gamma, 2Balpha, 3a, 3b, 4E6*	*pAbp, GlyRS, CG7482*	*Hrb87F*	*YTHDF*
Unclear	*4EHP*	*eIF6*	*DENR, syp*	
Control Phenotype		*eIF2D*	*Sqd, heph, Unr, La, Larp*	*YTHDC1, SAM-S*

Table 2 Legend: Translation initiation factor complex categorisation according to knockdown phenotype

This table highlights the classification of each factor according to their knockdown phenotype. Phenotypes are classed as either i) Loss of Zfh1-positive CySCs, ii) gain of Zfh1-positive CySCs or iii) Control-like phenotype, outlined in the first column. Knocking down components of complex eIF4F, resulted in varied phenotypes, but were generally classed as loss of Zfh1-expressing CySC phenotypes, indicating a role in CySC self-renewal. Factor knockdowns associated with complexes, eIF1, eIF2 and eIF3, generally resulted in gain of Zfh1-expressing CySC phenotype, most showing a role in cyst cell differentiation. Cap-independent and universal factor knockdowns displayed varied phenotypes, but were generally classed as gain of Zfh1-positive CySC phenotypes, indicating a role in cyst cell differentiation.

Table 3: Translation initiation factor knockdown phenotype scoring results

Knockdown	Sample Size	CySC Present (%)	Cyst Cells Present (%)	Hub Present (%)	CySC Away from Hub (%)	Normal GSC Differentiation (%)
Control	22	100	100	100	0	100
eIF4E1	17	47	100	52	0	5
eIF4EHP	16	100	100	100	0	87.5
eIF4E3	21	100	100	100	0	100
eIF4E4	30	100	100	100	0	100
eIF4E6	15	100	100	100	20	78
eIF4A	17	17	100	88	0	0
eIF4B	16	100	100	100	0	100
eIF4G	20	33	100	6	0	0
eIF4H1	19	100	100	100	0	100
eIF4H2	16	100	100	100	0	100
eIF3b	31	100	100	88	82	7
eIF3a	15	100	93	84	100	0
eIF1	18	100	100	100	16	100
eIF1A	16	100	68	100	100	0
eIF2alpha	16	100	100	100	86	6
eIF2gamma	15	100	100	100	60	0
eIF2Balpha	18	100	100	100	11	100
pAbp	25	100	100	100	95.6	n/a
GlyRS	15	100	100	100	40	n/a
eIF6	17	58	100	52	35	n/a
CG7482	17	100	100	100	70	n/a
eIF2D	16	100	100	100	0	100
DENR	18	100	100	100	5	n/a
sqd	16	100	100	100	0	n/a
heph	26	100	100	100	0	n/a
Unr	17	100	100	100	0	n/a
syp	21	100	100	90.4	26.3	n/a
Hrb87F	16	100	100	100	93.8	n/a
La	17	100	100	100	0	n/a
Larp	16	100	100	100	0	n/a
YTHDF	15	100	100	100	73	0
YTHDC1	15	100	100	100	0	100
SAM-S	16	100	100	100	0	100

Table 3 Legend: Translation initiation factor knockdown phenotype scoring results

This table provides details of the selection criteria for our classification method and a percentage summary of each knockdown phenotype, according to i) Zfh1-expressing CySC presence/absence, ii) Eya-expressing cyst cell presence/absence, iii) Fas3-expressing hub presence/absence, iv) Zfh1-expressing CySCs outside the niche and v) GSC differentiation.

3.4 Validating the screen

3.4.1 The requirement for eIF4A in self-renewal is confirmed by eIF4A-mutant MARCM clones

In order to confirm the primary results of the screen I harnessed the powerful genetics of *Drosophila* and used the Mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999) to generate mutant clones for two independent *eIF4A* alleles, $eIF4A^{1006}$ and $eIF4A^{1013}$. This technique enabled me to generate labelled homozygous mutant CySCs in an otherwise heterozygous organism (Lee and Luo, 1999). Using RNAi, I observed that eIF4A knockdown led to a lack of CySC self-renewal (see Fig. 7B). By generating mutant clones, I could assess whether CySCs that are homozygous mutant for *eIF4A* could self-renew to confirm this finding with an independent method. I investigated the phenotype of eIF4A mutant clones at 2 days post-clone induction (dpci) and 7dpci. The 2dpci time point allowed me to ascertain that mutant clones were being induced. By 7dpci, if these cells display a self-renewal defect, one would expect to no longer find labelled cells in the niche. Therefore, the number of testes containing labelled mutant CySCs at 7dpci compared to a control indicates the self-renewal capacity of the mutant cells.

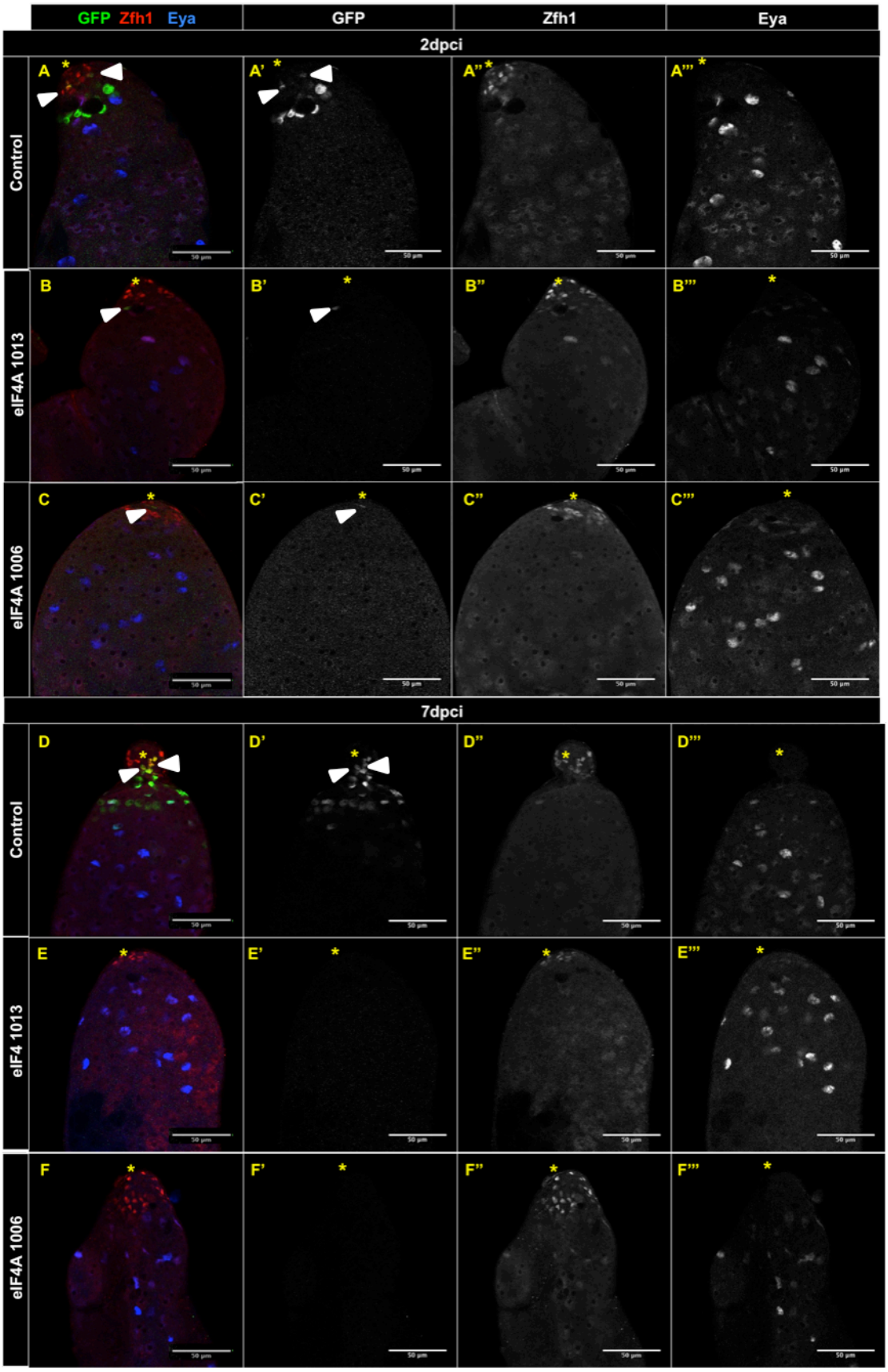
I crossed $eIF4A^{1013}$, FRT^{40A} mutant males with *y,w,hs-Flp,Tub-Gal4,UASnlsGFP/FM7;FRT40A Tub, Gal80* virgins and incubated these at 25°C, along with a control cross where I generated wild-type labelled cells using the same FRT site. I then selected and collected male progeny every 2 days and heat-shocked the flies for 1 hour in a 37°C water bath before returning them to 25°C. I immunostained the testes at 2 and 7 dpci, using antibodies against Green Fluorescent Protein (GFP) (clones), Eya (cyst cells) and Zfh1 (CySCs). Finally, I scored for the presence of CySC clones, cyst clones, GSC clones and differentiated GSC clones, all marked by the expression of GFP.

I found that the frequency of testes with control CySC clones decreased slightly over 7dpci (see Fig. 14A-A'', Table 3), which is to be expected due to neutral competition (Amoyel et al., 2014). By contrast I saw a rapid loss of CySC clones homozygous for $eIF4A^{1013}$, from 40% at 2dpci (see Fig. 14B-B'', 14G), to 0% at 7dpci (see Fig. 14E-E'', 14G, Table 4). Similarly, the frequency of CySC clones homozygous mutant for $eIF4A^{1006}$ decreased from 5% at 2dpci (see Fig. 14C-C'', 14G), to 0% at 7dpci (see

Fig. 14F-F’’’, 14G). For data summary please see Table 4 of Appendix 1. The low recovery rate at 2dpci, could be due to the low clone induction or the fact the clones had already begun differentiating.

These results show that eIF4A mutant CySC clones differentiate rapidly, causing a complete loss of these mutant stem cells from the niche in both cases after 7dpc (see Fig. 14G). Due to rapid differentiation of mutant CySCs, I conclude that eIF4A is required for CySC self-renewal. This reinforces and confirms the results from our screen, proving that eIF4A is required in CySCs autonomously for self-renewal.

Figure 14: eIF4A-mutant MARCM CySC clones are lost after 7dpci



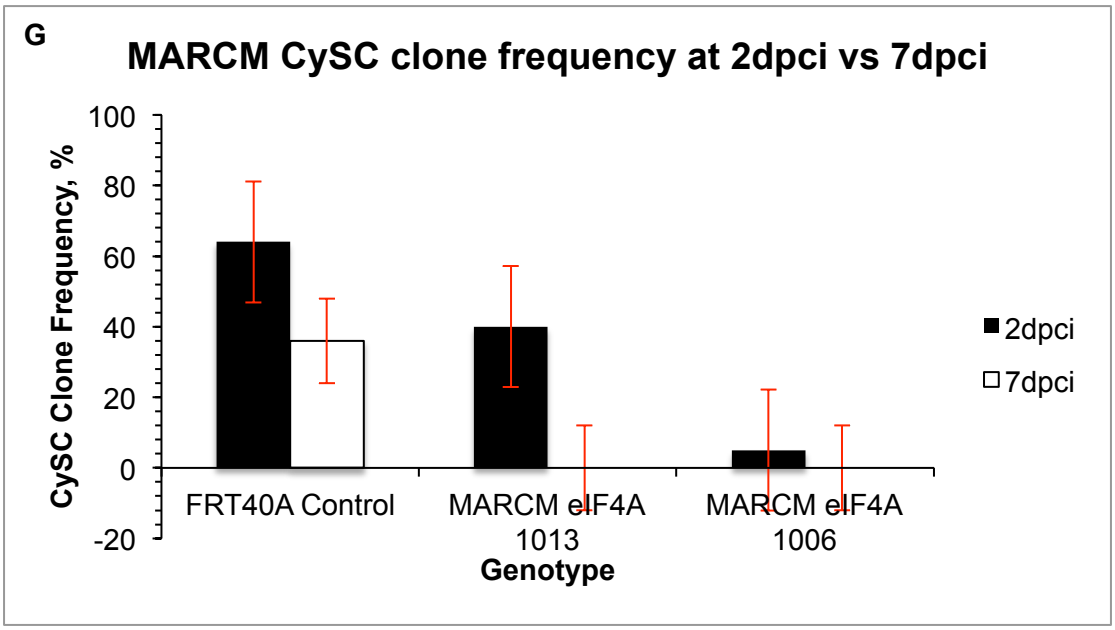

G
MARCM CySC clone frequency at 2dpci vs 7dpci
CySC Clone Frequency, %
100
80
60
40
20
0
-20
FRT40A Control
MARCM eIF4A 1013
MARCM eIF4A 1006
Genotype
2dpci
7dpci

***Figure 14 Legend:** eIF4A-mutant MARCM CySC clones are lost after 7dpci*

MARCM CySC clones express nuclear GFP, which was labelled using anti-GFP antibody (green). The cyst lineage expresses Tj, therefore anti-Tj antibody was used to identify both CySCs and cyst cells (blue) depending their proximity to the hub (asterisk). Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody was used to identify CySCs (red). All images have 50um scale bars. Control FRT40A (A-A'") nuclear GFP-labelled clones highlighted Zfh1-expressing CySCs (A, A', A", green/red, arrows) in the niche (asterisk) at 2dpci. Control FRT40A (D-D'") nuclear GFP-labelled clones also highlighted Zfh1-expressing CySCs (D, D', D", green/red, arrows) in the niche (asterisk) at 7dpci. Nuclear GFP-labelled eIF4A^{1013} mutant clones (B-B'") highlighted Zfh1-expressing CySCs (B, B', B", green/red, arrows) in the niche (asterisk) at 2dpci. However, no GFP-labelled Zfh1-expressing CySCs (E, E', E") were observed at 7dpci. This suggests that self-renewal is lost in response to eIF4A disruption, implying a role for eIF4A in CySC self-renewal maintenance. Nuclear GFP-labelled eIF4A^{1006} mutant clones (C-C'") highlighted very few Zfh1-expressing CySCs (C, C', C", green/red, arrows) in the niche (asterisk) at 2dpci. However, no GFP-labelled Zfh1-expressing CySCs (F, F', F") were observed at 7dpci. This suggests that self-renewal is lost in response to eIF4A disruption, implying a role for eIF4A in CySC self-renewal maintenance. (G) A bar chart highlighting the difference in CySC clone frequency for each genotype at 2dpci (black) and 7dpci (white).

3.4.2 Knocking down canonical initiation factors affects translation rates in CySCs

In order to investigate the correlation between screen phenotypes and translation levels, I performed a protein synthesis assay on each of the RNAis with the strongest phenotypes, including; eIF4E1, eIF4A, eIF4G, eIF3a and eIF2alpha.

I investigated the translation rates of CySCs, daughter cells and GSCs, by conducting a protein synthesis assay using O-propargyl-puromycin Click-iT reaction (Liu *et al.*, 2012). Collecting male progeny from the identical crosses to the screen, I only incubated them at 29°C for 2 days to prevent loss of CySCs or excess CySCs from impairing comparisons between CySCs and differentiating cyst cells. I used the same protocol as in our previous OPP investigations. CySCs and daughters cells were stained using antibodies against Traffic Jam (Tj), and cell membranes and the hub using discs large (Dlg). I scored CySCs and differentiating cyst cells based on proximity to the hub and used the membrane staining to outline individual cells, as previously described. I then measured and compared OPP incorporation of individual cells by using ImageJ's Mean Gray Value (MGV) setting.

Firstly, I wanted to see whether translation had been affected by knocking down initiation factors. This was achieved by measuring OPP incorporation for CySCs and GSCs in individual testes. Due to *Gal4* being regulated by a CySC driver, the RNAis are only expressed in the cyst lineage. Therefore, I used GSC translation rates as an internal control, assuming that knocking down factors in CySCs would have a non-cell autonomous impact on the translation rates of GSCs. I measured OPP incorporation for CySCs and GSCs, then performed an unpaired t-test to assess the significance of the data. Using GSCs as an internal control means I was able to eliminate changes in staining intensity that were due to differences in staining between individual samples. I found that in control testes (see Fig. 15), there was no significant difference between translation rates in CySCs and adjacent GSCs, with a p value> 0.5 (Student's T-test, CySCs vs GSCs). If translation rates were affected by knocking down the above-mentioned factors, I expected to see a significant difference in translation rates. Indeed, knocking down eIF4E1, eIF4A and eIF3a led to a significant decrease in the OPP incorporation in CySCs compared to GSCs in the same testis (see Fig. 15). This suggested that overall translation in CySCs had been reduced by knocking down these factors. Although I saw a decrease in OPP incorporation in CySCs of eIF4G and eIF2alpha knockdowns, the difference was not

statistically significant compared to control (see Fig. 15). Therefore, I cannot be sure that translation was affected by knocking down these factors. For data summary please see Table 5 of Appendix 2.

Secondly, I investigated whether the difference in OPP incorporation seen in CySCs and their daughters was affected by manipulating individual initiation factors. I identified and measured Mean Gray Value in CySCs and neighbouring cyst cells that did not contact the hub in individual testes, then compared and ran an unpaired t-test on the data. As previously stated, I observed significantly higher OPP incorporation results (p value< 0.04, Student's T-test, CySCs vs daughters) in OregonR CySCs compared to their daughter cells, suggesting that CySCs synthesise more protein than their differentiating daughter cells (see Fig. 5). In all knockdown experiments, I observed that differences between the OPP incorporation of CySCs and differentiating cyst cells were abolished (see Fig. 16). This suggests that translation initiation factors regulate the observed difference between CySC and daughter cell translation rates. For data summary please see Table 6 of Appendix 2.

Overall these data support the hypothesis that translation initiation is a key step in controlling translation rates, both in absolute terms, and to control differences in translation between cells of different fates.

Figure 15: GSC vs CySC Translation Rates in Translation Initiation Factor Knockdowns

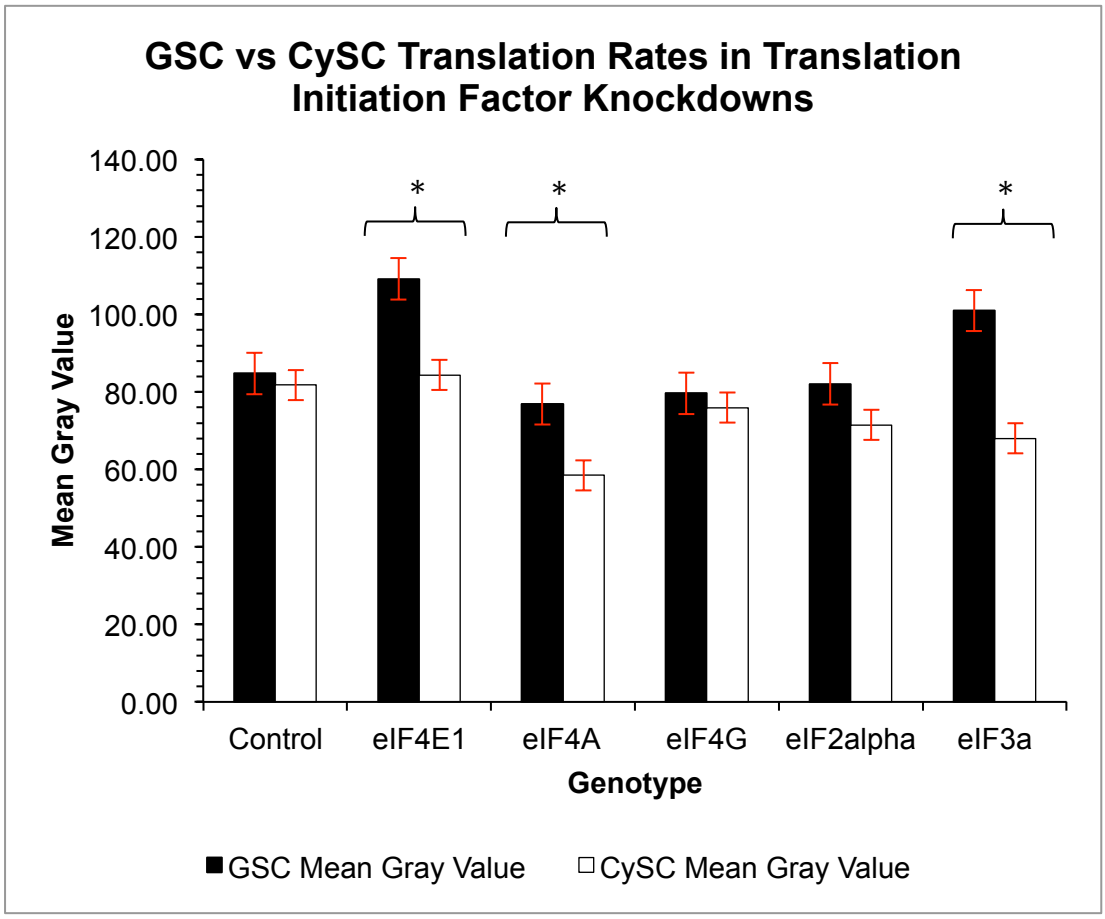


Figure 15 Legend: GSC vs CySC Translation Rates in Translation Initiation Factor Knockdowns

The bar chart displays the (MVG), used to determine the incorporation value of OPP, in individual GSCs (black) compared CySCs (white) of the same testis, with standard error bars (red). OPP incorporation was used to assess translation rates in different cells staged at 2 days. In control testes translation rates were similar in GSCs (black) and CySCs (white). Knocking down eIF4E1 led to significantly lower (P value < 0.002, Student's T-test, CySCs vs GSCs) in CySCs (white) compared to GSCs (white) in the same testis, suggesting that translation in CySCs is affected when manipulating eIF4E1. Knocking down eIF4A led to significantly lower (P value < 0.05, Student's T-test, CySC vs GSCs) in CySCs (white) compared to GSCs (white) in the same testis, suggesting that translation in CySCs is affected when manipulating eIF4A. Knocking down eIF4G led to similar translation rates in GSCs (black) and CySCs (white) in the same testis, suggesting that translation in CySCs is not affected when manipulating eIF4G. Knocking down eIF2alpha led to similar translation rates in GSCs (black) and CySCs (white) in the same testis, suggesting that translation in CySCs is not affected when manipulating eIF2alpha. Knocking down eIF3a led to significantly lower (P value < 0.004, Student's T-test, CySC vs GSCs) in CySCs (white) compared to GSCs (white) in the same testis, suggesting that translation in CySCs is affected when manipulating eIF3a.

Figure 16: CySC vs Cyst Cell Translation Rates in Translation Initiation Factor Knockdowns

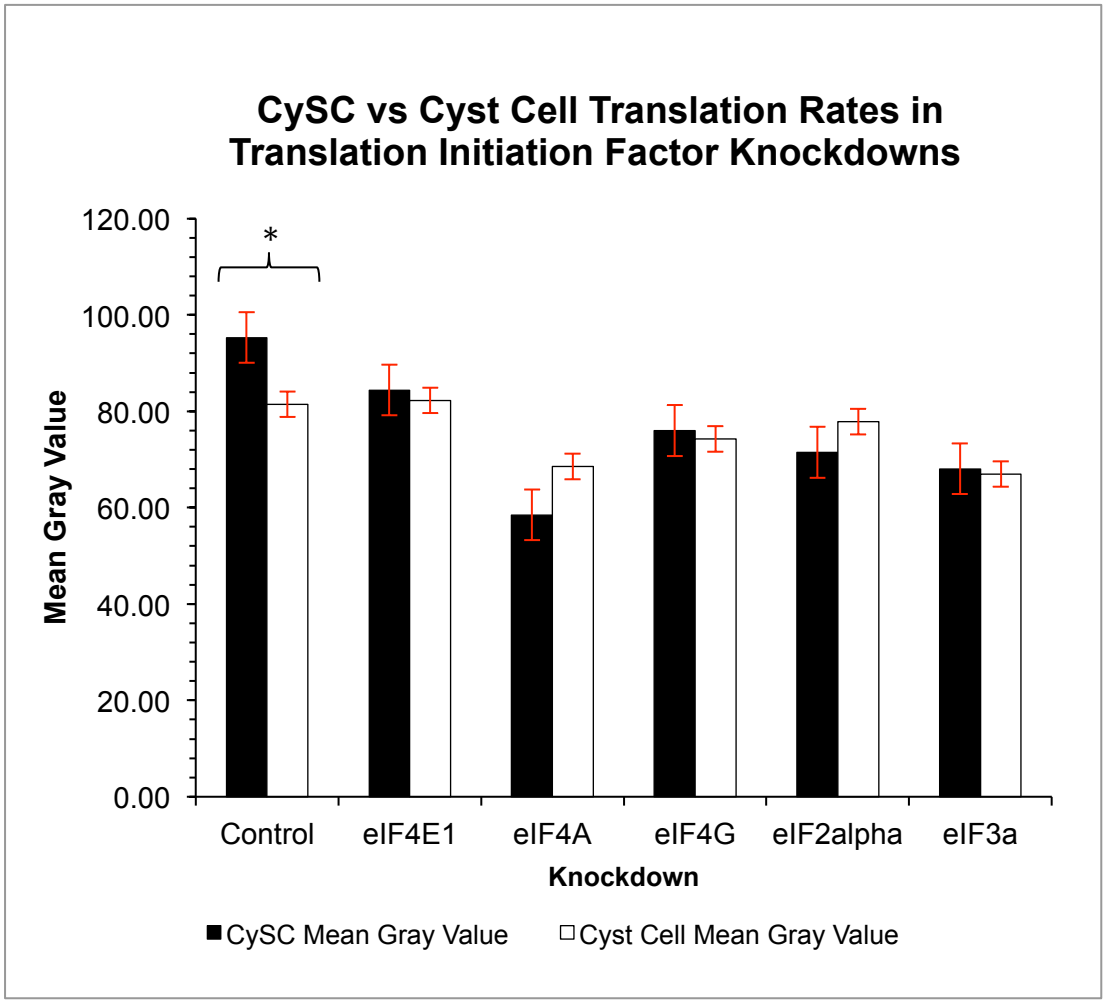


Figure 16 Legend: CySC vs cyst cell translation rates in translation initiation factor knockdowns

The bar chart displays the Mean Gray Value (MVG), used to determine the incorporation value of OPP, in individual CySCs (black) compared differentiating daughters/cyst cells (white) of the same testis, with standard error bars (red). OPP incorporation was used to assess translation rates in different cells. Translation rates were significantly higher (P value < 0.04, Student's T-test, CySC vs cyst cells) in CySCs (black) compared to their daughter cells/cyst cells (white). Knocking down eIF4E1, eIF4A, eIF4G, eIF2alpha and eIF3a led to the differences in OPP incorporation of CySCs (black) and their differentiating daughters/cyst cells (white) being abolished. This suggests that translation initiation factors regulate the observed difference between CySC and daughter cell translation rates.

3.5 Tor versus Translation

3.5.1 Inhibiting Tor rescues eIF4F components knockdown phenotypes

Firstly, I investigated this by feeding eIF4F RNAi expressing flies the TORC1 inhibitor rapamycin, to assess whether TORC1 operates upstream or downstream of eIF4A. I set up identical crosses to the screen (see section 3.2) using eIF4F RNAi lines; eIF4E1, eIF4A, eIF4G alongside a *Tj-Gal4; Tub, Gal80* control subjected to the same conditions. I fed half the progeny rapamycin-containing food and the other regular food. After 10 days at 29°C, I stained the testes using Zfh1 (CySCs), Fas3 (Hub), Eya (cyst cell) and Topro (GSC differentiation) antibodies. I scored each phenotype for the presence of i) CySCs, ii) cyst cells, iii) hub, iv) CySCs outside the niche and v) normal GSC differentiation. Knocking down eIF4F components led to a complete depletion of Zfh1-expressing CySCs in the testes (see Fig. 17C, 17E, 17G). By contrast, feeding control flies rapamycin-containing food led to ectopic CySCs being observed away from the hub and a lack of Eya-expressing cells, recapitulating previous work showing that Tor activity is required for CySC differentiation (Amoyel et a., 2016) (see 17B-B'''). Testes from flies expressing eIF4G and eIF4E1 RNAi that were fed Rapamycin-containing food had a striking rescue of Zfh1-expressing cells compared to the RNAi alone. Zfh1-expressing cells were present in 100% of samples (see Table 7) in eIF4E1 (see Fig. 17C-D''') and eIF4G (see Fig. 17G-H''') knockdown with Rapamycin, compared to 0% in the knockdowns alone (see Table 6). Similarly, in 90% of testes expressing RNAi against (see Table 7) eIF4A (see Fig. 17E-F'''), Zfh1-expressing cells were present when Tor activity was blocked by Rapamycin feeding. Tor is therefore epistatic to eIF4F in CySC differentiation.

Figure 17: Feeding rapamycin to eIF4F knockdown flies rescues the eIF4F knockdown phenotype

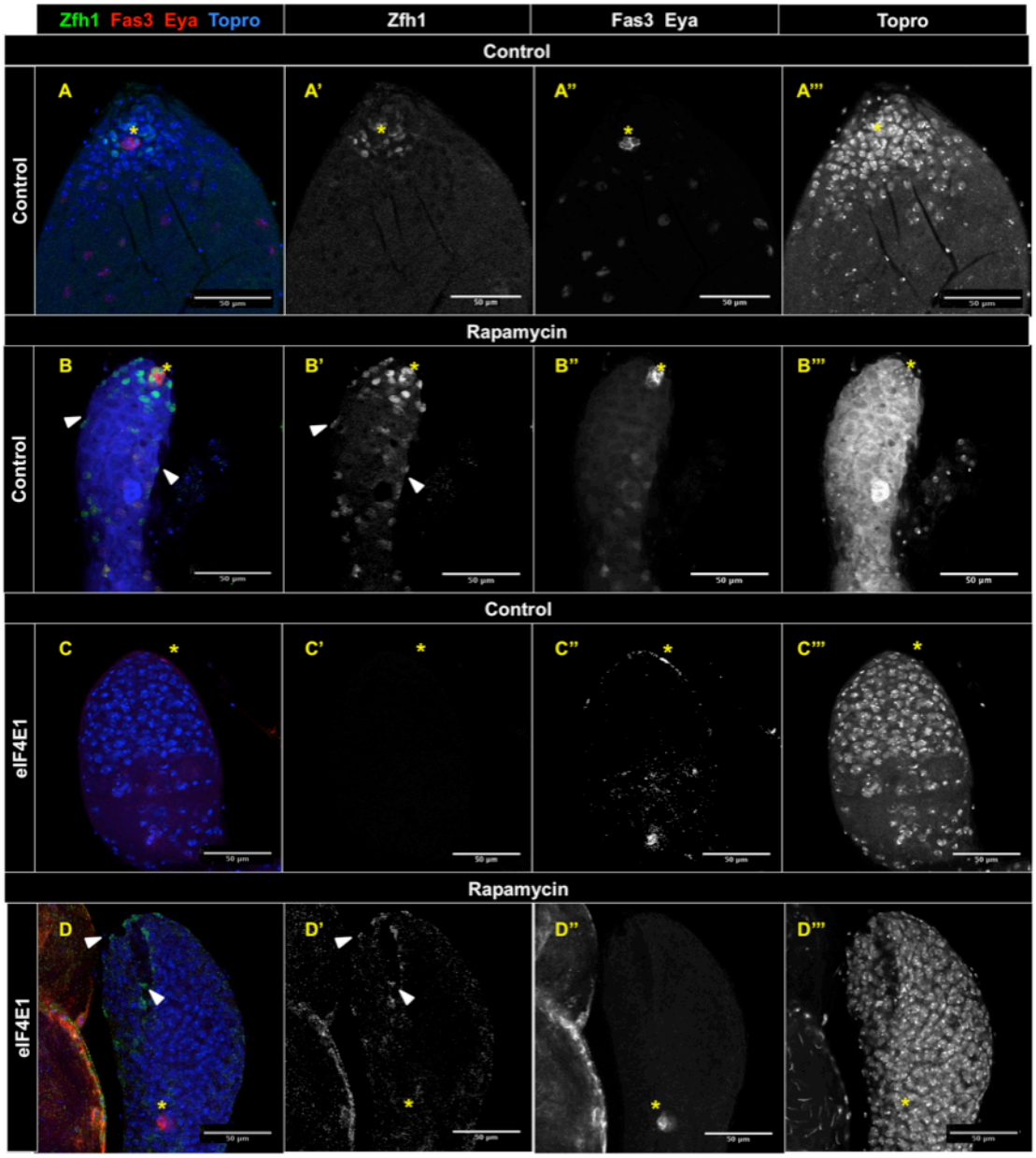


Figure 17: Feeding rapamycin to eIF4F knockdown flies rescues the eIF4F knockdown phenotype

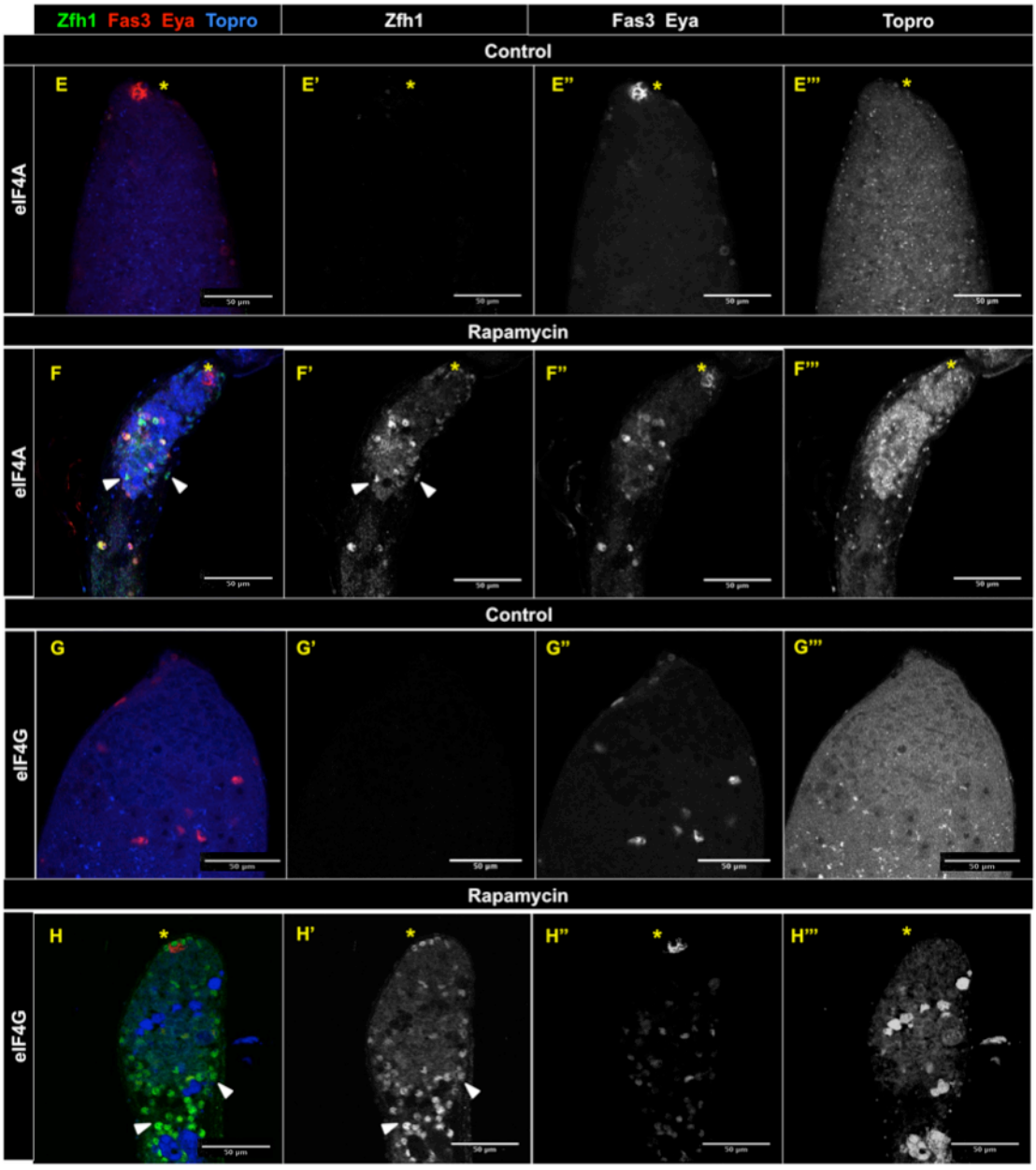


Figure 17 Legend: Feeding Rapamycin to eIF4F knockdown flies rescues the eIF4F knockdown phenotype

Zfh1 is expressed in CySCs, therefore anti-Zfh1 antibody (green) was used to identify CySCs. Eya is expressed in differentiating cyst cells, therefore anti-Eya antibody (red) was used to identify cyst cells. Testis hubs express Fas3, thus anti-Fas3 antibody was used to identify hub (red, asterisk). Cell nuclei were stained using Topro antibody (blue). All images have 50um scale bars and display the testis apex. Control testes of flies that were fed control (A-A''') had all cell types present (A, green), including Zfh1-expressing CySCs (A, A', green), Eya-expressing cyst cells (A'', red) and a Fas-3 expressing hub (A'', asterisk). Zfh1-expressing cells were observed within the niche (A, A', green) and Topro staining revealed normal GSC differentiation, due to a specific staining pattern (A, A''', blue). Feeding control flies rapamycin (B-B''') led to Zfh1-expressing CySCs increasing in number (B, B', green) and being found outside the niche (asterisk)(A, A', green, arrows). This suggests that inhibiting PI3K/Tor activity prevents differentiation, as observed in Amoyel et al., 2016. Knocking down eIF4E1 in flies on control food (C-C'''), led to a complete depletion of Zfh1-expressing CySCs (C, C', green), with only Eya-expressing cyst cells (C, C'', red) remaining and abnormal GSC differentiation (C, C''', blue). This mimicked the results of the screen. Feeding eIF4E1 knockdown flies rapamycin (D-D'''), recues the phenotype. Zfh1-expressing CySCs increased in number (D, D', green) and were found outside the niche (asterisk) (D, D', green, arrows), resembling the phenotype of PI3K/Tor fed control testes (A-A'''). The rescue suggests that Tor may behave downstream of eIF4E1. Knocking down eIF4A in flies on control food (E-E'''), led to a complete depletion of Zfh1-expressing CySCs (E, E', green), with only Eya-expressing cyst cells (E, E'', red) remaining and abnormal GSC differentiation (E, E''', blue). This mimicked the results of the screen. Feeding eIF4A knockdown flies rapamycin (F-F'''), recues the phenotype. Zfh1-expressing CySCs increased in number (F, F', green) and were found outside the niche (asterisk) (F, F', green, arrows), resembling the phenotype of PI3K/Tor fed control testes (A-A'''). The rescue suggests that Tor may behave downstream of eIF4A. Knocking down eIF4G in flies on control food (G-G'''), led to a complete depletion of Zfh1-expressing CySCs (G, G', green), with only Eya-expressing cyst cells (G, G'', red) remaining and abnormal GSC differentiation (G, G''', blue). This supported the results of the screen. Feeding eIF4G knockdown flies rapamycin (H-H'''), recues the phenotype. Zfh1-expressing CySCs increased in number (H, H', green) and were found outside the niche

(asterisk) (H, H', green, arrows), resembling the phenotype of PI3K/Tor fed control testes (A-A'''). The rescue suggests that Tor may behave downstream of eIF4G.

Table 7: Inhibiting Tor rescues eIF4 knockdown phenotypes

Knockdown	Food	Sample Size	CySC Present (%)	Cyst Cells Present (%)	Hub Present (%)	CySC Away from Hub (%)	Normal GSC Differentiation (%)
Control	C	13	100	100	100	0	100
Control	R	10	100	70	100	100	0
eIF4E1	C	15	42	100	71	0	0
eIF4E1	R	15	100	50	92	100	0
eIF4A	C	10	40	90	100	0	0
eIF4A	R	10	100	70	90	90	0
eIF4G	C	21	42	100	85	0	0
eIF4G	R	17	100	64	100	100	0

Table 7 Legend: Inhibiting Tor rescues eIF4 knockdown phenotypes

This table contains a percentage summary of each knockdown phenotype when fed control food (C) and rapamycin-containing food (R). The phenotypes were scored according to i) Zfh1-expressing CySC presence/absence, ii) Eya-expressing cyst cell presence/absence, iii) Fas3-expressing hub presence/absence, iv) Zfh1-expressing CySCs outside the niche and v) GSC differentiation.

3.5.2 p4E-BP levels are lower in eIF4F component knockdowns

Although Tor inhibition can block the loss of differentiation observed upon knockdown of eIF4F components, this could be due to Tor and eIF4F acting either in a linear pathway, or in parallel to affect CySC fate. To distinguish between these possibilities, I stained eIF4F knockdowns for phosphorylated 4E-BP (p4E-BP), which is a direct target of TORC1. If eIF4F directly inhibits Tor then one would expect to see increased p4E-BP levels when eIF4F components were knocked down. I selected and aged males for 2 days at 29°C and stained eIF4E1, eIF4A and eIF4G knockdowns using antibodies against p4E-BP (Amoyel, Hillion, *et al.*, 2016), Tj (cyst lineage) and Fas3 (hub). I scored phenotypes as i) control-like, ii) increased p4E-BP expression and iii) decreased p4E-BP expression (see Table 8). In a control testis (see Fig. 18A-A'''), p4E-BP was detected in Tj-positive cells 1 cell diameter from the hub, implying that Tor activity, as reported by p4E-BP expression, was low in CySCs and high in differentiating cyst cells. This expression pattern matched well with the pattern published previously (Amoyel, Hillion, *et al.*, 2016). In testes in which eIF4E1 (see Fig. 18B-B'''), eIF4A (see Fig. 18C-C''') and eIF4G (see Fig. 18D-D''') was knocked down, p4E-BP expression levels were decreased overall compared to control testes (see Table 8). Expression levels were decreased such that the pattern observed in control testes was affected, where p4E-BP expression levels appeared equally low in both CySCs and cyst cells. To confirm these results with an independent approach, I examined p4E-BP levels in *eIF4A* mutant clones. I generated $eIF4A^{1013}$ CySC MARCM clones and analysed them at 2dpci as mutant CySCs were only recovered at this time point. Testes containing mutant clones were immunostained with p4E-BP. Mean Gray Values of CySC mutant clones were compared to neighbouring CySCs as an internal control to correct for variations in staining intensity across samples. I stained testes using anti GFP (clone), Tj (cyst lineage) and p4E-BP antibodies. Mean Gray Values of p4E-BP intensity were reduced in $eIF4A^{1013}$ CySC mutant clones compared to neighbouring control CySCs (see Fig. 19). This result matches that observed with RNAi knockdowns and indicates that p4E-BP is indeed reduced in cells lacking eIF4A. Since p4E-BP is a reporter for Tor activity, these results suggest that Tor activity is reduced in response to knocking down eIF4F. However, the previously described functional experiments (see Fig 17, Table 7, section 3.5.1) indicate that Tor activity is required downstream of eIF4F loss. Although these experiments lead to contrasting conclusions on the relationship between Tor and eIF4F, it is likely that p4E-BP is not a good reporter for

Tor activity in this context and therefore experiments should be repeated using an alternative reporter before concluding the state of the relationship.

In summary, depletion of Zfh1-expressing CySCs observed upon eIF4F knockdown is rescued in almost 100% of testes (see Table 7) when inhibiting Tor, suggesting that Tor is epistatic to eIF4F. However, staining for the Tor reporter p4E-BP in eIF4F knockdowns and eIF4A^{1013} MARCM clones, showed a reduction of p4E-BP levels (see Fig. 19), suggesting a reduction in Tor activity, or, more likely, that p4E-BP is not accurately reporting Tor activity in this context.

Figure 18: p4E-BP levels are lower in eIF4F component knockdowns

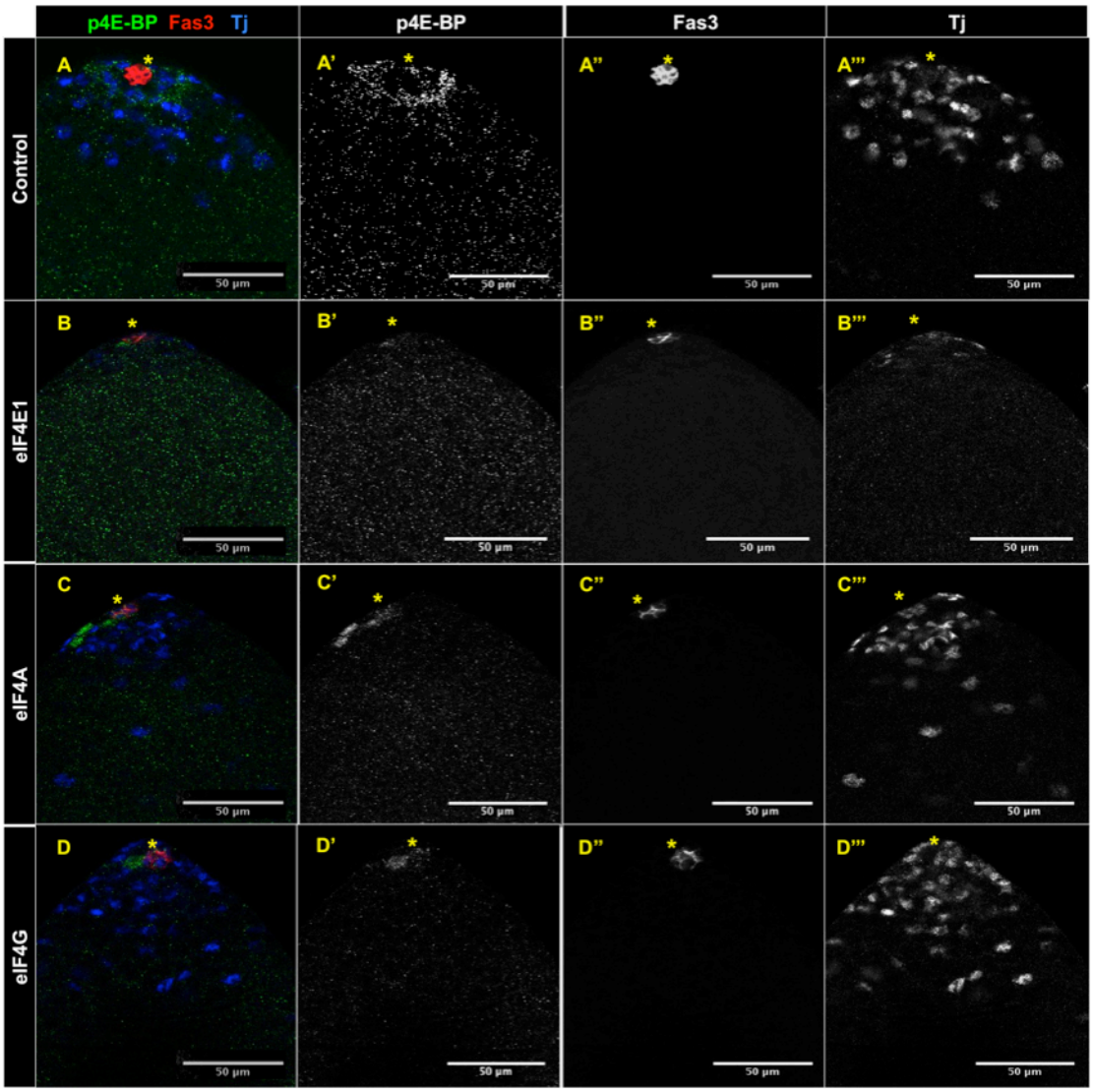


Figure 18 Legend: p4E-BP levels are lower in eIF4F component knockdowns

p4E-BP is a target of TORC1 and often used as a reporter protein for the PI3K/Tor pathway. Therefore, anti-p4E-BP antibody was used to assess the activity of the pathway in knockdown testes (green). Hub cells express Fas3, therefore anti-Fas3 antibody was used to label the hub (red, asterisk). Tj is expressed in the cyst lineage, therefore anti-Tj antibody was used to identify CySCs and cyst cells (blue) depending on their proximity to the hub (red, asterisk). In control testes (A-A'''), p4E-BP was detected in a ring pattern around the hub (asterisk), particularly strong in Tj-expressing cells 1 cell diameter from the hub (asterisk)(A, A', green). This suggests that Tor activity was low in CySCs and high in differentiating cyst cells. Knocking down eIF4E1 (B-B''') led to complete loss of p4E-BP staining (B, B', green) in Tj-expressing cells (B, B''', blue). This suggests that Tor activity is reduced when knocking down eIF4E1. Knocking down eIF4A (C-C''') led to complete loss of p4E-BP staining (C, C', green) in Tj-expressing cells (C, C''', blue). This suggests that Tor activity is reduced when knocking down eIF4A. Knocking down eIF4G (D-D''') also led to complete loss of p4E-BP staining (D, D', green) in Tj-expressing cells (D, D''', blue). This suggests that Tor activity is reduced when knocking down eIF4G.

Figure 19: Control vs Mutant CySC p4E-BP Levels

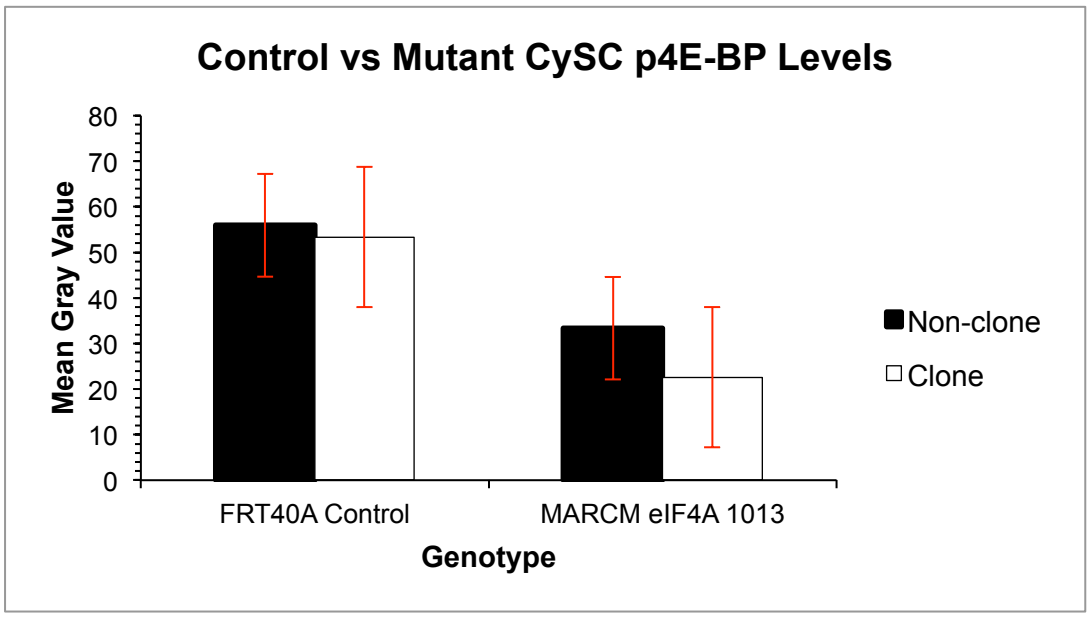


Figure 19 Legend: p4E-BP levels are reduced in eIF4A^{1013} mutant CySC clones compared to non-clone CySCs

The bar chart displays the Mean Gray Value (MVG), used to determine p4E-BP staining intensities; in individual non-clone CySCs (black) and clone CySCs (white), with standard error bars (red). p4E-BP levels were lower in eIF4A^{1013} mutant clone CySCs (black) non-clone CySCs in the same testis (white).

Table 8: p4E-BP levels were lower in eIF4F knockdown testes

Knockdown	Age (days)	Sample Size	p4E-BP levels Control (%)	p4E-BP levels increased (%)	p4E-BP levels decreased (%)
Control	2	26	100	0	0
eIF4E1	2	3	0	0	100
eIF4A	2	6	0	0	100
eIF4G	2	12	0	0	100

Table 8 Legend: p4E-BP levels were lower in eIF4F knockdown testes

This table contains a summary of p4E-BP staining data in control and eIF4F knockdown testes. The data is presented as a percentage summary of each knockdown phenotype, according to i) control p4E-BP staining, ii) increased p4E-BP staining and iii) decreased p4E-BP staining.

3.6 The regulation of self-renewal factor Stat92E by eIF3/2alpha

3.6.1 Knocking down eIF3/2alpha leads to ectopic Stat92E in GSCs and CySCs

Next, I investigated further the phenotypes of eIF3 and eIF2alpha knockdowns. Due to their apparent role in promoting differentiation, I sought to measure the expression of self-renewal factor Signal transducer and activator of transcription factor 92E (Stat92E) in each knockdown. Stat92E is one of the main self-renewal factors that maintain CySCs. When JAK/STAT signalling is low, transcription factor Stat92E is degraded, but is stabilised when there is high signalling through Upd expression on hub cells (Leatherman and Dinardo, 2008; Amoyel and Bach, 2012). Therefore, Stat92E protein is used to report on JAK/STAT signalling levels. Since, JAK/STAT signalling promotes CySC self-renewal and has been known to induce ectopic stem cells away from the hub, I investigated JAK/STAT signalling levels in eIF3 knockdown testes, which resulted in ectopic Zfh1-expressing CySCs. I crossed eIF3/2alpha RNAi male flies with *Tj-Gal4; Tub, Gal80* virgins and aged progeny for 10 days at 29°C, along with *Tj-Gal4;Tub,Gal80* control flies. I immunostained using Stat92E (Flaherty et al., 2010), Tj (cyst lineage) and Vasa homolog (germline) antibodies. In the control I observed Stat92E in CySCs and GSCs close to the niche (see Fig. 20A-A'', Table 9). In testes in which eIF3a subunits were knocked down, ectopic Stat92E expression in CySCs were observed outside the niche in 71% (5/9) of testes (see Fig. 20B-B''', Table 9). Similarly, I observed ectopic Stat92E expression in undifferentiated GSCs in 71% (5/9) of testes. I observed similar results in 100% (2/2) of eIF2alpha knockdown testes (see Fig. 20C-C''', Table 9). Despite the small sample sizes, I hypothesised that Stat92E expression could be regulated downstream of eIF3/eIF2alpha. Since I also observed ectopic Stat92E non-autonomously in the germline, it seems likely that a JAK/STAT pathway ligand was produced ectopically in testes in which eIF3 was knocked down. However increased Stat92E is not necessarily the cause of ectopic CySCs being seen in eIF3/2 knockdowns. It could also be a consequence, and one would need to carry out epistasis experiments to assess that. One possibility is that eIF3-depleted cells are expressing Upd ligands in response to stress, a known response in other tissues in the fly. In these examples, stressed or dying cells undergo Jnk activity and produce Upd ligands downstream of Jnk (Worley, Alexander and Hariharan, 2018). To address this possibility I stained flies of the same genotypes with phosphorylated Jun N-terminal kinase (pJnk) antibody, a reporter for stress related pathways (Wang,

Bohmann and Jasper, 2003). I observed increased levels of pJnk in CySCs, in 90% (9/10) of eIF3 RNAi testes (see Fig. 21B-B’’’, Table 10) and in 100% (5/5) of eIF2alpha testes (see Fig. 21C-C’’’, Table 10), compared to the control (see Fig. 21A-A’’’, Table 10). These results are consistent with my hypothesis and suggest that both pathways are activated ectopically in eIF3/2 knockdowns. Future experiments will test whether stress-induced JNK is a response to knockdown conditions or defects in translation and increase the sample size of this experiment.

Figure 20: eIF3 and eIF2 alpha knockdowns lead to ectopic Stat92E expression in the cyst lineage and germline

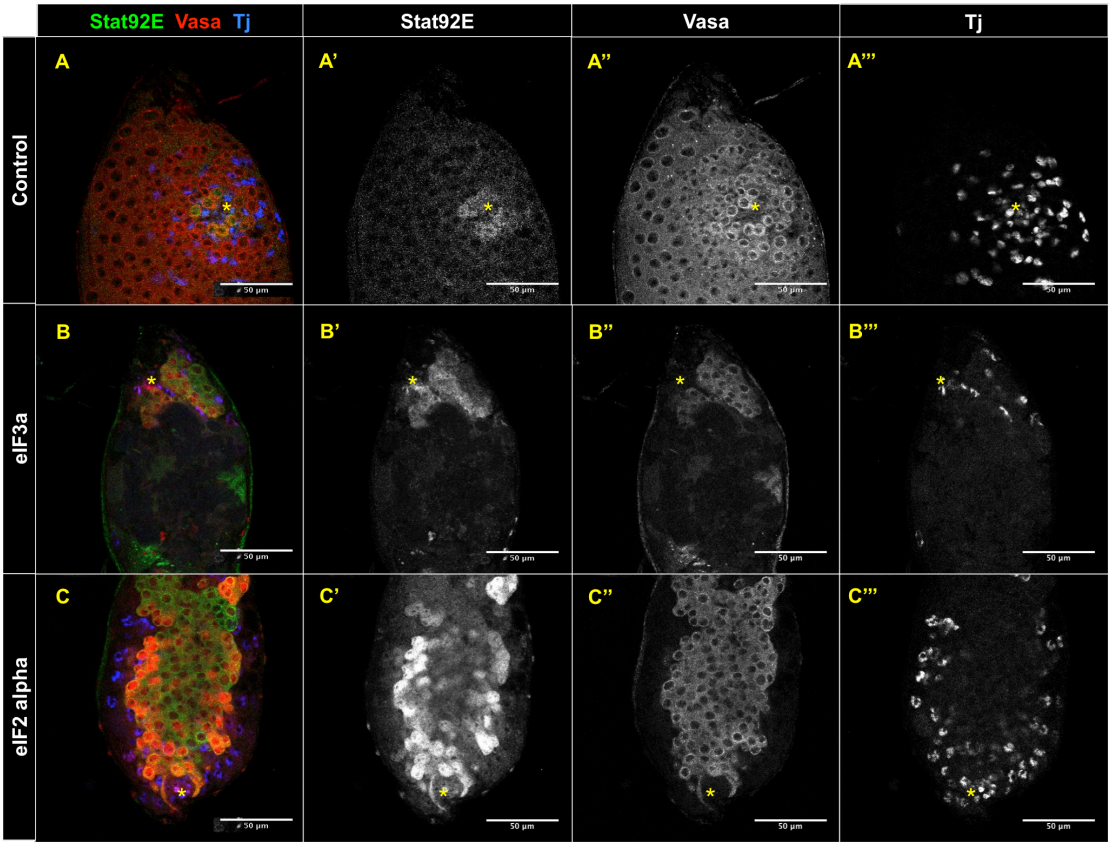


Figure 20 Legend: eIF3a and eIF2alpha knockdowns lead to ectopic stat expression in the cyst lineage and germline

Stat92E is a core self-renewal factor in the cyst lineage, which is used to assess self-renewal ability and report on JAK/STAT activity. Here, anti-Stat92E antibody is used to investigate JAK/STAT activity (green) in eIF3/2alpha knockdowns. Tj is expressed in the cyst lineage and so anti-Tj antibody is used to identify CySCs and cyst cells (blue) depending on their proximity to the hub (asterisk). Anti-vasa antibody is used to label cell membranes (red). All images have 50um scale bars, displaying the testis apex. In control testes (A-A'''), Stat92E (A, A', green) was only detected in Tj-expressing cells (A, A''', blue) in contact with the hub (asterisk). This suggests that JAK/STAT is active in self-renewing CySCs. Knocking down eIF3a (B-B''') led to ectopic Stat92E expression (B, B', green) in both Tj-expressing cells (B, B''', blue) and Tj-negative germline cells (B, B'', red), away from the hub (asterisk). Knocking down eIF2alpha (C-C''') also led to ectopic Stat92E expression (C, C', green) in both Tj-expressing cells (C, C''', blue) and Tj-negative germline cells (C, C'', red), away from the hub (asterisk). This suggests that regulation of Stat92E could be downstream of eIF3/2.

Table 9: eIF3a and eIF2alpha knockdowns lead to ectopic stat expression in the cyst lineage and germline

Knockdown	Age (days)	Sample Size	Control Stat92E levels (%)	Ectopic Stat92E (%)
Control	10	6	100	0
eIF3a	10	7	29	71
eIF2alpha	10	2	0	100

Table 9 Legend: eIF3a and eIF2alpha knockdowns lead to ectopic stat expression in the cyst lineage and germline

This table contains a summary of Stat92E staining data in control and eIF3/2 knockdown testes. The data is presented as a percentage summary of each knockdown phenotype, according to i) control Stat92E staining, ii) ectopic Stat92E staining.

Figure 21: eIF3 and eIF2alpha knockdowns leads to increased pJnk expression in ectopic CySCs

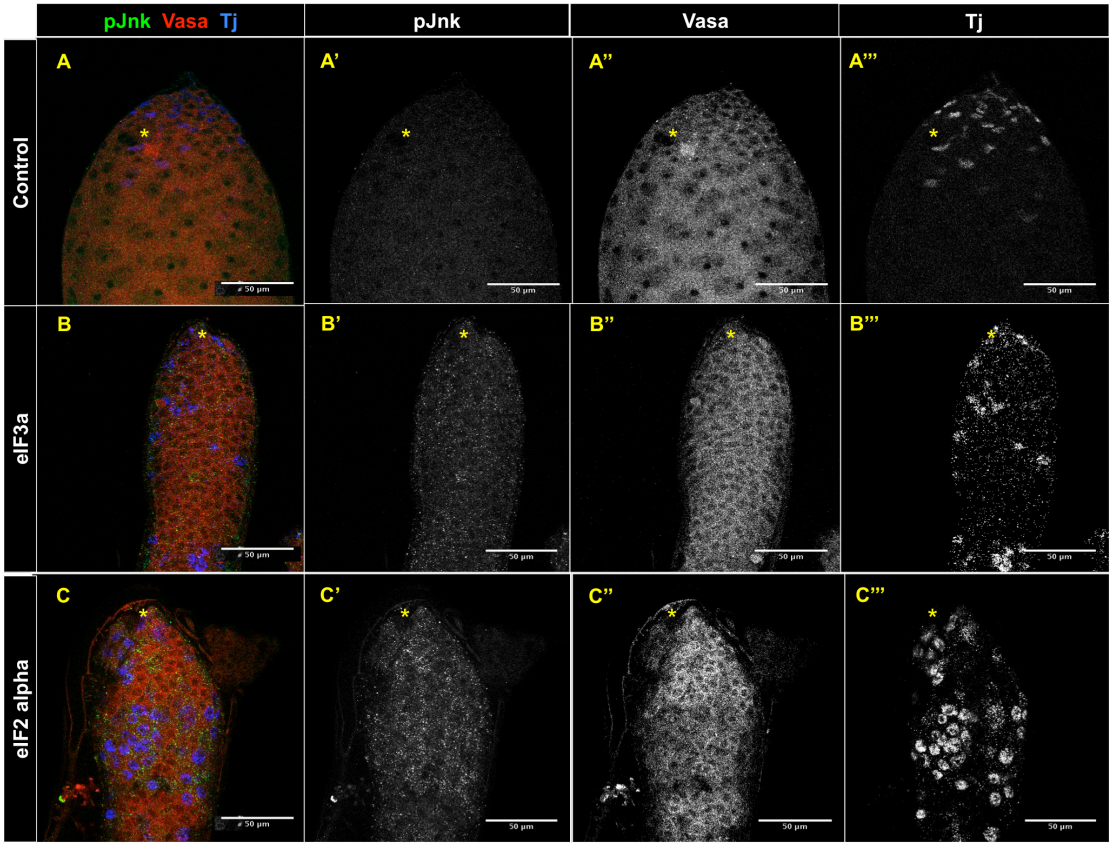


Figure 21 Legend: eIF3 and eIF2alpha knockdowns leads to increased pJnk expression in ectopic CySCs

pJnk is used as a reporter for Jnk activity, therefore pJnk-antibody was used to assess Jnk activity (green) in the testis. Tj is expressed in the cyst lineage and so anti-Tj antibody is used to identify CySCs and cyst cells (blue) depending on their proximity to the hub (asterisk). Anti-vasa antibody is used to label cell membranes (red). All images have 50um scale bars, displaying the testis apex. In control testes (A-A'''), pJnk was not detected (A, A', green) in any cell lineage (A, A''', blue), implying that pJnk is not expressed in response to knockdown conditions. Knocking down eIF3a (B-B''') led to increased pJnk levels to be detected ectopically (B, B', green) in Tj-expressing cells (B, B''', blue) and Tj-negative germline cells (B, B'', red), away from the niche (asterisk). Knocking down eIF2alpha (C-C''') also led to increased pJnk levels to be detected ectopically (C, C', green) in Tj-expressing cells (C, C''', blue) and Tj-negative germline cells (C, C'', red), away from the niche (asterisk). This suggests that pJnk levels increase in response to knocking down eIF3/2.

Table 10: eIF3 and eIF2alpha knockdowns leads to increased pJnk expression in ectopic CySCs

Knockdown	Age (days)	Sample Size	Control pJnk levels (%)	pJnk levels increased (%)	pJnk levels decreased (%)
Control	10	9	100	0	0
eIF3a	10	10	10	90	0
eIF2alpha	10	5	0	100	0

Table 10 Legend: eIF3 and eIF2alpha knockdowns leads to increased pJnk expression in ectopic CySCs

This table contains a summary of pJnk staining data in control and eIF3/2 knockdown testes. The data is presented as a percentage summary of each knockdown phenotype, according to i) control pJnk staining, ii) increased pJnk staining and iii) decreased pJnk staining.

4.0 Discussion

4.1 Translation is important in CySC fate

The aim of this project has been to understand how translation initiation factors control stem cell fate in the *Drosophila* testis niche. I have confirmed preliminary results from the Amoyel lab that demonstrated CySCs have higher translation rates than their differentiating daughters. I carried out an RNAi screen to identify potential roles for translation initiation factors in CySC fate. Knocking down eIF4F components resulted in the loss of CySCs through differentiation, while knocking down other components of other initiation complexes such as eIF3 or eIF2alpha resulted in ectopic CySCs. eIF4A knockdown results were reflected in MARCM studies using *eIF4A* mutant alleles. Mutant clones of *eIF4A* were unable to self-renew at the niche and differentiated rapidly. The contrasting functions of eIF4F with the other initiation complexes suggest a model in which CySC fate is be regulated by alternative translation initiation mechanisms. eIF4F complex is not required during differentiation, however eIF3 is, implying that translation initiation during differentiation is cap-independent and thus could depend on IRES. In addition to canonical initiation factors, I also knocked down factors associated with alternative translation initiation mechanisms, such as IRES. Overall, the RNAi screen suggested a role for non-canonical factors in promoting CySC differentiation, with some (pAbp, GlyRS, CG7483, Hrb87F and YTHDF) mimicking the eIF3 knockdown phenotype. Investigating protein synthesis rates in potent knockdowns revealed a significant impact on translation when comparing GSCs and CySCs in eIF4E1, eIF4G and eIF2alpha knockdowns. When comparing OPP incorporation of CySCs versus differentiating daughters, the differences in rate were abolished compared to the control. This suggests that translation is regulated during initiation and supports a role for translation in CySC fate. Since eIF4F knockdowns and previously published Tor loss-of-function experiments showed opposing results, I tested the relationship between Tor activity and eIF4F. Feeding Rapamycin to eIF4F RNAi males resulted in a rescue of the eIF4F phenotype implying that Tor is epistatic to eIF4F. Finally, I investigated whether knocking down eIF3 and eIF2alpha led to defects in differentiation and excess stem cells because of increased self-renewal signalling. Here, I stained for readout of active JAK/STAT signalling, Stat92E protein. The results showed increased levels of Stat92E in both knockdown conditions, suggesting these factors play a role in regulating the expression or activity of the JAK/STAT pathway. Overall, our results suggest that translation plays an important role in controlling stem cell fate in the testis, and that the regulation of translation initiation is the important stage for this fate determination.

4.2 Translation initiation factor RNAi screen

In order to determine the success of the screen, results should be supplemented by additional experiments that reliably portray the function of the gene, such as MARCM clones. RNAis are often used in screens as they are readily available, easy to breed into fly lines and generally reliable as preliminary results. A disadvantage to using RNAis is the possibility of them not working or having off-target effects (Cherry *et al.*, 2005; Heigwer, Port and Boutros, 2018). Additionally, an RNAi screen is not a perfect method to assess the roles of proteins due to low levels of *Gal4* expression that can lead to inefficient RNAis and off-target effects (Heigwer, Port and Boutros, 2018). Many non-canonical factor knockdowns produced a control phenotype, which meant that I could not draw any conclusions as to whether these factors play a role in CySC fate. A negative result from an RNAi (here, a control phenotype) does not necessarily imply that the gene plays no role, but warrants to repeat the experiment and confirm knockdown using an antibody or Real-time Polymerase Chain Reaction (qPCR) (Holmes *et al.*, 2010). Alternatively, one can use several different RNAi lines targeting the same gene but with different sequences. I aimed to circumvent these issues by knocking down several factors belonging to the same complex, such as eIF4F. Where I recorded the same phenotypes when knocking down factors belonging to the same complex, I could rely on these results. This strengthened my confidence in my data and allowed me to trust the RNAi phenotypes are real and not random off-target effects. I also confirmed the phenotype of eIF4A knockdown by generating eIF4A mutant clones using MARCM and observing a similar result. I suggest using mutant alleles to confirm other screen hits, especially where they do not act as part of a clear complex, such as ITAFs. Other limitations to the screen include the genetic background of the RNAi lines and the use of “blank” controls. Ideal control experiments for the screen would have included scrambled RNAi lines or individually recommended control lines, i.e. TRiP RNAi control lines from Bloomington or GD RNAi control lines from Vienna Stock Centre. Using specific control lines would provide a more accurate control phenotype to compare the screen knockdowns to. When sourcing RNAi stocks from Vienna Drosophila Resource Centre, I aimed to obtain only GD lines, where insertions are P-element based transgenes with random insertion sites. However, occasionally GD lines were not available and I sourced KK lines, with an insertion of the RNAi hairpin contruct which is based in landing site 30B and/or 40D. KK lines with a 40D landing site are

notorious for off-target effects and causing non-specific phenotypes when crossed with certain Gal4 drivers (Green *et al.*, 2014). Therefore, it would be imperative to verify the results of the screen and use available KK library controls.

4.2.1 Differentiation or Death

It is vital to mention that with the current experimental design, an observed loss of stem cells could be due to differentiation or cell death, in response to drastic, downstream effects caused by the RNAi. The design does not allow to distinguish between the two outcomes. In the text, I assume that the cells are lost to differentiation due to Eya-expressing cells of the cyst lineage still being present after the induction of the RNAi and incubation 29°C (see Table 3). Additionally, by generating *eIF4A*^{1013} and *eIF4A*^{1006} MARCM mutant clones, I was able to trace cells of the cyst lineage. There was a rapid loss of CySC clones through differentiation between 2dpci and 7dpci (see Fig. 14). This was identified due to Eya-expressing and GFP-positive cyst cell clones being observed in *eIF4A*^{1013} and *eIF4A*^{1006} MARCM testes at both time points. Although lineage tracing through MARCM is one method to determine whether the CySCs indeed differentiate in factor RNAi knockdowns, it would be imperative to investigate whether CySCs are dividing, differentiating or dying. I would suggest conducting 5-ethynyl-2'-deoxyuridine (EdU) staining assays in RNAi knockdowns staged for 2-3 days at 29°C (Daul, Komori and Lee, 2010). This would allow one to assess whether CySCs are dividing and differentiating into cyst cells. Additionally, it would be of interest to stain RNAi knockdowns for apoptotic markers, such as Terminal deoxynucleotide transferase-mediated dUTP end labelling (TUNEL), (Sarkissian *et al.*, 2014). This method would allow one to label apoptotic CySCs where DNA has been cleaved by endonucleases and apoptosis has been initiated (Sarkissian *et al.*, 2014). Finally, it would be important to generate MARCM clones for most important translation initiation factors, such as eIF4G and eIF3a, both to supplement the results of the screen and to trace the cyst lineage.

4.2.2 eIF4F promotes a different fate to eIF3/2/1

At first, the screen investigates the roles of canonical 5’ cap-dependent translation initiation factors. Other than identifying a role for translation in CySC fate, the contrasting phenotypes of eIF4F knockdowns compared to other complexes

associated with 5' cap-dependent initiation of translation, i.e. eIF1, eIF2 and eIF3 are perplexing (see Tables 2 & 3). They suggest that different complexes may individually be involved in different cell fates, i.e. eIF4F promotes self-renewal and eIF3/2/1 promote differentiation. This was also confirmed by generating MARCM clones with mutated eIF4A, where I saw the loss of CySC clones through differentiation (see Table 4). Preliminary data in the lab also showed that *eIF3-S9* mutant clones were unable to differentiate, validating the results of the RNAi screen that eIF3 and eIF4F components act in opposite ways on CySC self-renewal. An explanation for this could be the use of alternative initiation mechanisms of translation in differentiating cyst cells, which do not require the cap-binding eIF4F complex Although 5' cap-dependent translation initiation is the most common and well-understood mode of initiation, there are at least 5 other methods that have been identified. These methods include Internal ribosomal entry site (IRES), 5' UTR m^6A translation, YTHDF1 m^6A translation, Ribosome shunting and RAN translation (Lasko *et al.*, 2005; Jackson, Hellen and Pestova, 2010; Mitchell and Parker, 2015; Yoffe *et al.*, 2016). The first 3 methods mentioned use other factors involved in cap-dependent translation such as the eIF3 complex components. Translation initiation requires the recruitment and binding of ribosomal units to the mRNA strand, which is commonly achieved through 5' cap binding to eIF4F complex, followed by recruitment of eIF3 and eIF2 components with the ribosome (Sonenberg and Hinnebusch 2009). However, translation initiation can also be achieved independently of the eIF4F complex, as with IRES, where recruitment is possible through interaction of mRNA with eIF3 complex or directly with the 40S ribosomal subunit (Sonenberg and Hinnebusch, 2007). Sonenberg and Hinnebusch also alluded to the idea of roles for 5' cap-independent modes of translation initiation mechanisms in *Drosophila* development in their 2007 review. More research reaffirms the importance of IRES in development, where IRES-mediated translation is important for Hox gene expression, to pattern the mammalian body (Xue *et al.*, 2015). Since eIF4F factor knockdowns suggest a role in self-renewal, I hypothesise that CySCs may regulate translation in a cap-dependent manner and change their translation programming to cap-independent methods upon differentiation.

The screen included knockdowns of factors involved in non-canonical translation initiation, including IRES-mediated translation. RNAi knockdowns form a first step in developing our understanding of the regulation of translation in the CySC lineage. However, using alternative and more accurate methods such as generating MARCM clones for non-canonical and canonical factors will shed light on this issue. Such

methods would allow one to investigate the effects of gene loss in individual cells, in a control environment.

4.2.3 eIF4E6 is not a traditional 5' cap-dependent initiation factor

Upon knocking down eIF4 complex components, including eIF4E paralogues, I found that eIF4E6 showed a role in cyst cell differentiation. This result contrasted with the effect of knocking down other eIF4E paralogues, which displayed a role in CySC self-renewal. However, previous research has shown that different isoforms of eIF4E have different eIF4G binding abilities, with eIF4E6 forming no interaction with eIF4G (Hernández *et al.*, 2005). Hernández et al. showed that eIF4E6 and eIF4EHP are unable to bind eIF4G and 4E-BP and act as negative regulators of cap-dependent translation. This raises the question as to whether eIF4E6 could promote cap-independent translation initiation by binding the 5' cap but not eIF4G and therefore decreasing cap-dependent translation initiation. Although this could explain the different phenotype of eIF4E6 knockdown compared to other eIF4E paralogues, it also conveys the complexity of translational regulation that may be involved in CySC fate. eIF4E6 may bridge the differences between 5' cap-dependent and 5' cap-independent translation initiation, and act as a translational regulator in differentiating cyst cells.

eIF4E6 could be an interesting factor to further explore by looking at other RNAi knockdowns and by using MARCM to generate homozygous mutant clones. It would also be interesting to investigate the phenotype of knocking down both eIF4E6 and eIF4G to understand the relationship between these two factors and their role in CySC fate.

4.2.4 Differentiating cyst cells use an alternative mechanism of translation initiation compared to their CySC parents

In addition to the well-characterised initiation factor components, I knocked down factors, selected from literature, which had been found to be associated with alternative methods of translation initiation (Marygold, Attrill, and Lasko 2017; Lasko 2000; S. F. Mitchell and Parker 2015; Komar and Hatzoglou 2011). The hypothesis was that knocking down non-canonical factors would result in phenotypes mimicking

that of knocking down eIF3/2/1 and I found this for a subset of the knockdowns, specifically; pAbp, GlyRS, CG7483, Hrb58F and YTHDF.

Knocking down pAbp causes the strongest phenotype with ectopic CySCs found in 95% (23/25) of testes (see Table 3). This result is both expected and surprising as pAbp is a non-canonical factor but is also very important in cap-dependent translation initiation through interaction with eIF4G (Kahvejian *et al.*, 2005; Komar and Hatzoglou, 2011b; Mitchell and Parker, 2015). According to our RNAi phenotype pAbp is not necessary for self-renewal and therefore may not interact with eIF4F to promote that fate. This suggests that pAbp works in a non-canonical fashion to regulate CySC fate. Certain, recent literature supports such an idea, whereby pAbp is involved in non-canonical translation initiation mechanisms and recruits the ribosome via an eIF3-pAbp bridge to initiate IRES (Thakor *et al.*, 2017). Other literature suggests a less specific role for pAbp in IRES, in that it interacts differentially with ITAFs to promote this form of initiation (Komar and Hatzoglou 2011). Other possibilities include pAbp's role in mRNA stability, therefore regulating cleavage by endoribonucleases (Wang and Kiledjian, 2000). This role could regulate CySC gene expression programs at a pre-translational stage, thereby governing the expression of self-renewal or differentiation genes.

Knockdowns of other non-canonical factors GlyRS and CG7483 showed similar phenotypes. eIF6 knockdown led to an unclear phenotype. I recorded ectopic CySCs in 35% (6/17) of testes, but also recorded the absence of CySCs in others (42%) and additionally an absence of the hub in 48% of testes (See Table 3). This meant that eIF6 activity promotes differentiation in some testes and self-renewal in others. eIF6 has been identified as a proto-oncogene, having found to be overexpressed in certain human tumours (Brina *et al.*, 2015). The specific role of eIF6 is still under debate as it acts downstream of growth factor stimulation but has a complicated relationship with growth factor-sensitive pathways, such as Tor and c-myc (Brina *et al.*, 2015; Miluzio *et al.*, 2016). It could be that indeed eIF6 is acting through translation and other processes to regulate CySC fate. A study in 2015 suggested that eIF6 modulated myofibroblast differentiation at a transcriptional level of (TGF-beta1) expression, subsequently operating through TGF-beta/Smad signaling pathway. The paper also highlighted that eIF6 selectively regulates Wnt signaling and beta-catenin through protein synthesis (Ji *et al.*, 2008; Yang *et al.*, 2015). eIF6 is clearly an interesting contender in CySC fate and could be a bridge between different fates.

Knocking down ITAFs and IRES associated factors led to an eIF3-like phenotype, as well as unclear and control-like phenotypes. Knocking down Hrb87F led to the strongest phenotype with ectopic CySCs being recorded in 94% (15/16) of testes (see Table 3). Hrb87F, an hnRNP A1 homolog has been found to be an ITAF, promoting IRES-mediated translation of c-myc, Cyclin D1 and other genes, in methyltransferase knockout mice fibroblast cell lines (Gao, Dhar and Bedford, 2017). Knocking down Hrb87F suggests that it promotes differentiation. Since Hrb87F promotes cap-independent translation, its knockdown phenotype suggests that alternative translation mechanisms may indeed be promoting cyst cell differentiation, however more research is required to confirm this. The results thus follow with the hypothesis that alternate translation initiation mechanisms may regulate gene expression when changing stem cell fate.

When knocking down another ITAF, DENR, I did not see the same results. I classed the phenotype as unclear due to 1/18 testes having ectopic CySCs (see Table 3) (Komar and Hatzoglou 2011). It is likely that the phenotype of this testis is an anomaly, caused by an off-target of RNAi. However, DENR is one of the few established proteins that controls IRES initiation in *Drosophila* and it is worth investigating its role further. It would be recommended to use other available DENR RNAis before concluding whether DENR functions in controlling cyst cell differentiation.

Another knockdown leading to an unclear phenotype was that of the IRES regulator protein syp (McDermott *et al.*, 2014). Knocking down syp led to ectopic CySCs in 26% (5/21) of testes but also a loss of hub cells in 1 testis. This suggests that syp is involved in cyst cell differentiation, however may also maintain the hub. Although the result of losing hub cells was not replicated in other testes, it would be interesting to look at syp in more detail. Syncrip is a highly conserved RNA-binding protein belonging to the hnRNP family and has multiple roles in gene expression. These roles range from RNA maturation and trafficking to mRNA degradation and IRES translation initiation, (McDermott *et al.*, 2014; Santangelo *et al.*, 2016). With a variety of functions, it could explain a potential loss of hub cells and would be of interest to further understand the effects of non-canonical translation in CySC fate.

Among other non-canonical translation initiation mechanisms, m6A-modified mRNA mediated initiation requires recognition proteins (or "m^6A readers") to modulate its

activity (Mitchell and Parker, 2015). One recognition protein is YTHDF, which when knocked down leads to a gain of CySC phenotype, with ectopic CySCs recorded in 73% (10/15) of testes (see Fig. 13). YTHDF recognizes m6A-modified RNA, binds and recruits ribosomes to initiate translation (Mitchell and Parker, 2015; Cui *et al.*, 2017). Thus, YTHDF promotes cyst cell differentiation, which implies RNA methylation may serve as a regulatory mechanism in stem cell fate, favouring differentiation. RNA methylation has been shown to promote differentiation in mouse embryonic stem cells (Y. Wang *et al.*, 2014; Geula *et al.*, 2015) and *Drosophila* development (Hongay and Orr-Weaver, 2011; Mitchell and Parker, 2015), supporting a theory that RNA methylation may be used to favour pro-differentiation genes in CySCs. Recent research has also shown that flies undergo sex-transformation when lacking all m^6A modified RNA (Lence *et al.*, 2016). Recent work has shown that mechanisms exist to maintain male identity autonomously in adult CySCs. Loss of male identity in the cyst lineage leads to ectopic Zfh1-positive cells (Qian *et al.*, 2014). This would support a hypothesis that excess Zfh1 and lack of Eya in the case of YTHDF knockdowns is due to loss of male identity. This should be tested by analysing markers of male and female cell fate in these knockdowns.

Knocking down other proteins involved in mRNA methylation initiation, results in control-like phenotypes. However, one cannot conclude as to the role of the other proteins one testes, as the lack of phenotype may be due to ineffective RNAi knockdown. Other RNAi lines are available and should be tested to determine whether mRNA methylation plays a role in cyst cell differentiation.

The general trend of non-canonical factor knockdowns is to demonstrate a role in the differentiation of CySCs which supports a hypothesis suggesting stem cell fate is governed by alternative translation initiation mechanisms. Since non-canonical translation is not as efficient as canonical methods (Merrick, 2004), this would also explain the increased rate of protein synthesis in CySCs compared to their differentiating daughters (see Fig. 5). However, it would be vital to test those with phenotypes by generating homozygous mutant clones via MARCM. Considering pAbp's relationship with eIF4G it would be interesting to investigate their relationship further in the context of CySC fate by knocking down both in a singular model (Kahvejian *et al.*, 2005). Additionally, due to a possible interaction between pAbp and eIF3, it could be of interest to knock down eIF3 whilst overexpressing pAbp to see whether this would rescue the phenotype and shed light on the role of pAbp (Thakor

et al. 2017; Komar and Hatzoglou 2011). It would also be of interest to look at other RNAis of factors that demonstrated an unclear function, such as syp, DENR and eIF6. Since eIF6 has been identified to regulate TGF-beta1 transcription in myofibroblasts (Yang *et al.*, 2015), it would be of interest to knockdown both eIF6 and dawdle (TGF-beta1 homologue) to see whether eIF6's effect on dawdle is responsible for the RNAi phenotype. Many non-canonical factor knockdowns produced a control phenotype, which meant one could not conclude whether these factors played any role in CySC fate determination. However, I neither conclude a lack of function due to the inefficiency of RNAis (Qiu, Adema and Lane, 2005; Heigwer, Port and Boutros, 2018).

4.3 Investigating protein synthesis rates in factor knockdowns

In order to see whether the phenotypes observed in our RNAi screen were indeed correlated with an effect on translation, I investigated protein synthesis rates in knockdown CySCs. An O-propargyl-puromycin protein synthesis assay was conducted on knockdowns of eIF4E1, eIF4A, eIF4G, eIF3a and eIF2alpha. First, the translation rates of CySCs were compared to those of GSCs as an internal control to see whether translation rates had changed and, in some knockdowns, they were significantly reduced in eIF4E1, eIF4A and eIF3a knockdowns. This suggests that translation rates were affected by manipulating these factors. Translation rates in CySCs did not change significantly compared to those of GSCs in eIF4G and eIF2alpha knockdowns. A likely reason for the results of knocking down eIF2alpha is that there is insufficient data (see Table 5), from which one cannot draw a significant conclusion. It would be very important to repeat the assay for this knockdown before concluding anything. Our sample size for eIF4G knockdowns was sufficient, however there was no statistically significant decrease in OPP incorporation. This is surprising given eIF4G's central role in protein synthesis and the fact that eIF4F component knockdowns led to a strong and penetrant loss of CySCs (Prévôt, Darlix and Ohlmann, no date). The experiment could have been unsuccessful due to the RNAi not working, the timing between the temperature shift and dissection being insufficient to allow expression or the stain being of poor quality. To confirm this, one would have to repeat it and confirm efficient knockdown through qPCR. If it is indeed true that eIF4G knockdown does not significantly decrease translation, one explanation could be that eIF4G has roles outside of its canonical role in promoting cap-dependent translation initiation. My experiments show that Tor is epistatic to

eIF4G. If eIF4G regulates CySC fate through Tor and not translation, this may suggest a reason for a lack of change in translation rates. A necessary investigation to assess whether manipulating eIF4G can have an effect on translation would be to generate MARCM clones which are homozygous mutant for eIF4G and measure OPP incorporation in CySC mutant clones and control CySCs. Mutant alleles are more reliable that RNAi and so comparing the effect of translation in mutant clones and neighbouring control CySCs would provide one with a more reliable result.

4.4 eIF4F interacts with Tor to maintain CySC self-renewal

Published work has identified a role for eIF4A in inactivating TORC1 in response to amino acid starvation (Tsokanos *et al.*, 2016). Our finding that protein synthesis decreases in differentiating cells does not correlate with our current knowledge of PI3K/Tor activity in the CySC lineage, where PI3K/Tor activity is high in the differentiating cyst cells (Amoyel, Hillion, *et al.*, 2016). Indeed, increased PI3K/Tor activity should lead to increased protein synthesis as Tor is a major regulator of translation (Miron *et al.*, 2001; Showkat, Beigh and Andrabi, 2014). Tor activity should result in increased eIF4F activity, as Tor directly phosphorylates and inactivates 4E-BP, an inhibitor of eIF4F activity. I tested the genetic relationship between Tor and eIF4F in CySCs to determine whether Tor was epistatic to eIF4F. I inhibited both Tor and eIF4F by feeding Rapamycin to eIF4F knockdown flies. Additionally, I tested whether Tor activity was affected by eIF4F knockdown by staining for phosphorylated 4E-BP, a reporter for Tor activity. Feeding Rapamycin (Fig. 17) rescued the loss of Zfh1-positive CySCs observed upon eIF4F knockdown. This suggests that Tor acts downstream of eIF4F in CySCs and that these two factors act antagonistically on CySC fate. If eIF4F were to inhibit Tor this would explain why CySCs require eIF4F to maintain self-renewal and Tor to promote differentiation. The hypothesis is that eIF4F may directly inhibit Tor to maintain self-renewal of CySCs, and cease inhibiting Tor in differentiating cyst cells. My interpretation of the relationship between Tor and eIF4F may, however, be subject to the caveat of an imperfect control. Firstly, rapamycin treatment should have been negatively controlled with a vehicle treatment (ethanol) and secondly a specific control RNAi line should have been used. Other research also identifies a role for eIF4A directly inhibiting Bag of Marbles (Bam), a differentiation factor that is necessary and sufficient for GSC differentiation (Dobrikov *et al.*, 2014). eIF4E can inhibit Myc-dependent apoptosis, causing cell proliferation in growth factor-restricted

cells (Tan *et al.*, 2000). All of this implies that eIF4 may have specific downstream affects aside from “blind” translation initiation, which would explain why the complex has a contrasting function in cell-fate to other factors. However, while this hypothesis seems to explain the contrasting roles of eIF4F and Tor, it remains to be explained how the activity of eIF4F switches off in differentiating cyst cells to enable Tor activity.

Staining for phosphorylated 4E-BP as a means of assessing Tor activity when eIF4F complex members are knocked down, produced a surprising result (Fig. 18). Phosphorylated 4E-BP levels were reduced in eIF4F knockdowns, suggesting that removing eIF4F does not enhance Tor activity. This was reflected in the reduced p4E-BP levels in eIF4A mutant clones compared to control clones in a MARCM experiment (See Fig. 19, Table 8). 4E-BP ordinarily binds eIF4E and prevents its interaction with eIF4G, blocking translation (Miron, Lasko and Sonenberg, 2003). The block is removed through the phosphorylation of 4E-BP at specific residues by TORC1 (Miron, Lasko and Sonenberg, 2003; McCormick, Tsai and Kennedy, 2011), thus p4E-BP should be a reliable reporter for Tor activity. Since I observed 4E-BP staining in GSCs in these experiments, it seems likely that the reduction observed was not due to bad staining (Fig. 18). A possible explanation for these results is that 4E-BP expression is regulated by cap-dependent translation. Thus with eIF4F unable to assemble, 4E-BP is not synthesised leading to lower levels of 4E-BP. Another possibility is that Tor directly binds to eIF4F and since 4E-BP binds eIF4E, it is possible that the interaction between Tor and 4E-BP happens when 4E-BP is in a complex with eIF4F. Disrupting the complex in one way or another could thus disrupt the ability of Tor to find and phosphorylate 4E-BP. To investigate the result of the p4E-BP stains, one would need to stain for an alternative reporter protein, such as phosphorylated S6K, to establish whether Tor is inhibited by eIF4. It would also be important to knock down both Tor and eIF4G/E/A to repeat the Rapamycin inhibition of Tor with an independent method. It would also be recommended to introduce an appropriate negative control with a vehicle treatment (ethanol) to any similar experiment.

Overall, the results described here suggest that eIF4F and Tor are antagonistic in regulating CySC fate, and that Tor is epistatic to eIF4F. Since I do not see increased activity of a Tor reporter when eIF4F is knocked down, one cannot conclude as yet whether Tor and eIF4F act in parallel or in a linear pathway.

4.5 Stat92E is down-regulated by eIF3/2

I investigated whether eIF3/2 promoted differentiation through regulating Stat92E activity. Stat92E protein was used as a readout for the JAK/STAT pathway (Amoyel and Bach 2012; Leatherman and Dineardo 2008). I observed higher levels of Stat92E in ectopic CySCs when I stained for the factor in eIF3/2 knockdown flies (Fig. 20). Since Stat92E activity controls CySC fate directly (Leatherman and Dinardo, 2008; Amoyel and Bach, 2012), increased Stat92E in eIF3/2alpha knockdowns could be responsible for the presence of ectopic CySCs Ectopic Stat92E was also present non-autonomously in the germline when eIF3 and eIF2 were knocked down in the cyst lineage with Tj-Gal4. This suggests that the JAK/STAT activity seen is due to a secreted factor, as it is non-autonomous to the cyst lineage, in which the RNAi is solely expressed (Fig. 21). Published work in other in imaginal discs during development and in the gut during injury response in *Drosophila* imply that Upd ligands are produced in stressed cells in response to Jnk activity (Wang, Bohmann and Jasper, 2003; Worley, Alexander and Hariharan, 2018). A possible explanation is that stressed CySC undergo Jnk activity and subsequently express increased levels of Upd ligands that signal to activate the JAK/STAT pathway in neighbouring cells. Thus, I next sought to test Jnk activity and see whether it could be detected ectopically in eIF3/2 knockdowns. Jnk is increased in eIF3/2 knockdowns, which suggests that this pathway is also induced and would propose a possible explanation as to why Stat activity is higher in the germline as well as CySCs. To investigate whether Stat activity is a response driven by stress rather than eIF3/2 knockdown one could knockdown Jnk to see whether the same phenotype is achieved and investigate Stat92E levels in a knockdown of both Jnk and eIF3/2. Unfortunately, the sample size of these experiments is limited and repeating them would be advised confirm the results.

4.6 Conclusions

- Translation is important in CySC fate.
- CySCs synthesise more protein than their differentiating daughter cells.
- eIF4F is required in self-renewing CySCs.
- eIF3/2/1 is promotes cyst cell differentiation.
- Certain non-canonical factors promote cyst cell differentiation.

- A switch from cap-dependent to cap-independent translation may determine CySC fate.
- Tor appears epistatic to eIF4F.
- eIF3/2 regulate Stat92E expression.

5.0 References

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6.0 Abbreviations

5'UTR = 5' Untranslated Region

4E-BP = Eukaryotic Initiation Factor 4E Binding Protein

Abl = Abelson Tyrosine Kinase

Akt/Pkb = Protein Kinase B

Apaf-1 = Apoptotic protease activating factor 1

Arm = Armadillo

BAM = Bag of Marbles

Bloomington = Bloomington Drosophila Stock Centre

BMPs = Bone Morphogenetic Proteins

Cap = N7-methylated Guanosine 5' cap

CySCs = Cyst Stem Cells

DAP5 = Death-Associated Protein 5

DENR = Density Regulated protein

dpci = days post-clone induction

Dpp = Decapentaplegic

Dlg/DLG = Discs Large

dm = *Drosophila melanogaster*

EdU = 5-ethynyl-2´-deoxyuridine

EGF = Epidermal Growth Factor

EGFR = Epidermal Growth Factor Receptor

eIF1 = Eukaryotic Initiation Factor 1

eIF1A = Eukaryotic Initiation Factor 1A

eIF2alpha = Eukaryotic Initiation Factor 2 subunit alpha

eIF2Balpha = Eukaryotic Initiation Factor 2B subunit alpha

eIF2gamma = Eukaryotic Initiation Factor 2 subunit gamma

eIF2D = Eukaryotic Initiation Factor 2D/ ligatin

eIF3a (eIF3-S10) = Eukaryotic Initiation Factor 3 subunit a

eIF3b (eIF3-S9) = Eukaryotic Initiation Factor 3 subunit b

eIF4A = Eukaryotic Initiation Factor 4A

eIF4B = Eukaryotic Initiation Factor 4B

eIF4E1 = Eukaryotic Initiation Factor 4E paralogue 1

eIF4E3 = Eukaryotic Initiation Factor 4E paralogue 3

eIF4E4 = Eukaryotic Initiation Factor 4E paralogue 4

eIF4E6 = Eukaryotic Initiation Factor 4E paralogue 6

eIF4EHP = Eukaryotic Initiation Factor 4EHP

eIF4F/eIF4 = Eukaryotic Initiation Factor 4 Complex

eIF4G = Eukaryotic Initiation Factor 4G

eIF6 = Eukaryotic Initiation Factor 6

Eya = Eyes absent

Fas3 = Fasciclin III

Flp = Flippase

FRT = Short Flippase Recognition Target

Gal4 = Galactosidase induced genes transcription factor 4

Gal80 = Galactosidase induced genes transcription factor 80

Gal80^{ts} = Temperature Sensitive Galactosidase induced genes transcription factor 80

GB = gonialblast

GD = P-element RNAi Library

GFP = Green Fluorescent Protein

GlyRS = Glycyl-tRNA synthetase

GSC = Germline Stem Cell

GTP = Guanosine triphosphate

heph = hephaestus

hESCs = Human Embryonic Stem Cells

Hh = Hedgehog

hnRNP = Heterogeneous Nuclear Ribonucleoprotein

Hpo = Hippo

Hrb87F = Heterogeneous Nuclear Ribonucleoprotein at 87F

hs = heatshock

HSC = Haematopoietic Stem Cell

IRES = Internal Ribosomal Entry Site

I**TAF** = Internal Ribosomal Entry Site Trans-activating Factors

JAK/STAT = Janus Kinase-Signal Transducer and Activator of Transcription

Jnk/JNK = Jun N-terminal kinase

KK = phiC31 RNAi Library

La = La autoantigen protein

Larp = La related protein

M^{6}A = N6-methyladenosine

m^{7}G 5' Cap = N7-methylated Guanosine 5' cap

MARCM = Mosaic Analysis with a Repressible Cell Marker

MCT-1 = Multiple copies in T-cell lymphoma-1

Met-tRNA^{Met} = methylated tRNA

MGV = Mean Gray Value

mRNA = Messenger Ribonucleic Acid

nls = nuclear localization signal

OPP = O-propargyl-puromycin

p4E-BP = Phosphorylated Eukaryotic Initiation Factor 4E Binding Protein

pAbp/PABP = Poly A Binding Protein

PBS = Phosphate-buffered Saline

PBT = PBS Triton

PBTB = PBS-Triton with Bovine Serum Albumin

PFA = Paraformaldehyde

PI3K/Tor = Phosphatidylinositol 3-kinase and Target of Rapamycin

PIC = Pre-initiation Complex

pJnk/pJNK = phosphorylated Jun N-terminal Kinase

PTB = Polypyrimidine Tract-binding protein

PTEN = Phosphatase and tensin homolog

qPCR = Real-time Polymerase Chain Reaction

RAN = Repeat-association non-AUG

RHP = Restricted Haematopoietic Progenitor

RNA = Ribonucleic Acid

RNAi = Ribonucleic Acid Interference

Robo2 = Roundabout 2

S6K = S6 kinase

SAM-S = S-adenosylmethionine synthetase

Slit-Robo = Slit-Roundabout

SMAD = Homologies of SMA (small worm phenotype) and MAD (Mother against Decapentaplegic)

Spi = Spitz

Sqd = squid

ss = single stranded

Stat92E/Stat = Signal transducer and activator of transcription factor 92E

TGF-beta1 = Transforming Growth Factor beta1

Tj = Traffic Jam

Topro = Topro-3-Iodide

Tor = Target of Rapamycin

TORC1 = Target of Rapamycin Complex 1

TORC2 = Target of Rapamycin Complex 2

tRNA = Transfer Ribonucleic Acid

tub = tubulin

TUNEL = Terminal deoxynucleotide transferase-mediated dUTP end labeling

UAS = Upstream Activation Sequence

Unr = Upstream of N-Ras

uORF = Upstream Open Reading Frames

Upd = Unpaired

Vasa = Vasa Homolog

Vienna/VDRC = Vienna Drosophila Resource Centre

YTH = YT521-B homology

YTHDC1 = YT521-B homology domain containing 1

YTHDF = YT521-B homology N6-methyladenosine RNA binding protein

Zfh1 = Zinc finger homeodomain-containing protein

6.0 Appendix 1

***Table 4:** eIF4A-mutant MARCM CySC clones are lost after 7dpci*

MARCM Genotype	dpci (days)	Sample Size	CySC clone present (%)	Cyst cell clone present (%)	GSC clone present (%)	Gonialblast clone present (%)
FRT40A Control	2	30	64	44	62	59
eIF4A^{1013}	2	24	40	30	3	26
eIF4A^{1006}	2	18	5	11	0	21
FRT40A Control	7	39	36	66	70	63
eIF4A^{1013}	7	30	0	26	4	9
eIF4A^{1006}	7	19	0	0	0	0

***Table 4 Legend:** eIF4A-mutant MARCM CySC clones are lost after 7dpci*

This table contains a summary of MARCM clone frequency in control and eIF4A MARCM genotypes. The data is presented as a percentage summary of each phenotype, according to i) presence/absence CySC clones, ii) presence absence of cyst cell clones iii) presence absence of GSC clones and iv) presence/absence of gonialblast clones.

7.0 Appendix 2

Table 5: GSC vs CySC translation rates in translation Initiation factor knockdown

Knockdown	Age (days)	Sample Size	GSC MGV	CySC MGV	CySC / GSC MGV	Unpaired t-test result
Control	2	36	84.74	81.78	0.97	0.552
eIF4E1	2	36	109.17	84.37	0.78	**0.001**
eIF4A	2	16	76.87	58.47	0.77	**0.042**
eIF4G	2	35	79.62	75.97	0.98	0.319
eIF2alpha	2	5	82.07	71.45	0.87	0.172
eIF3a	2	17	101.00	68.03	0.77	**0.003**

Table 5 Legend: GSC vs CySC translation rates in translation Initiation factor knockdowns

This table contains a summary of the data collected from measuring Mean Gray Values (MVG) in GSCs and CySCs in translation initiation factor knockdowns. Mean Gray Values (MVG) were used to determine OPP incorporation, which in turn was used to identify translation rates in different cells.

Table 6: CySC vs cyst cell translation rates in translation Initiation factor knockdowns

Knockdown	Age (days)	Sample Size	CySC MGV	Cyst Cell MGV	CySC/ Cyst Cell MGV	Unpaired t-test result
Control	2	36	95.29	81.46	1.12	**0.038**
eIF4E1	2	36	84.37	82.27	1.05	0.584
eIF4A	2	16	58.47	68.51	0.91	0.090
eIF4G	2	35	75.97	74.24	1.04	0.783
eIF2alpha	2	5	71.45	77.83	0.93	0.546
eIF3a	2	17	68.03	66.98	1.05	0.850

Table 6 Legend: CySC vs cyst cell translation rates in translation Initiation factor knockdowns

This table contains a mean of the data collected from measuring Mean Gray Values (MVG) in CySCs and differentiating daughters/cyst cells in translation initiation factor knockdowns. Mean Gray Values (MVG) were used to determine OPP incorporation, which in turn was used to identify translation rates in different cells.